Parasitology (1960), **50**, 287–301
With 6 text-figures
Printed in Great Britain

Studies on Indian Rhynchobdellid leeches.* I

By SANJIB CHANDRA BAUGH, Ph.D.†
University of Lucknow, Lucknow, India

(*Received* 19 *May* 1959)

Our knowledge of the Indian Rhynchobdellid leeches is still in its infancy, and the little that we have, we owe to the researches of Harding, Kaburaki, and Moore. After the publication of the Hirudinea volume by Harding & Moore (1927) in the *Fauna of British India* Series, Bhatia (1930*a*, *b*, 1934, 1939), Chelladurai (1934) and Raj (1951, 1954*a*, *b*) have contributed to the systematics of this group. The present work has been undertaken with a view to advancing further our knowledge of Indian Rhynchobdellid leeches. In this work, the neurosomic theory of somite limits launched independently by Castle (1900) and Moore (1900) has been followed in counting the annular composition of somites. This paper, the first of the series, deals with the description of four Glossiphonid leeches, of which three are new to science, while the fourth one is of zoogeographical importance.

Placobdella horai‡ sp.nov. (Figs. 1*a*, 1*b*, 2*a*, 2*b*)

External morphology. The body (Fig. 1*a*) is thick, flattened and ovate-acuminate in shape, with crenated margins. On the dorsal side it is studded with numerous, small, closely-set papillae. The body is 5·9–11·6 mm. long, and its maximum width in the post-equatorial region is 1·8–3·8 mm. There is a pair of small cup-shaped eyes (Fig. 1*a*, 1*b*, 2, *e*.) on the second annulus. The eyes are close together and the concavity of each cup is directed antero-laterally. The ventral anterior sucker (Fig. 2*b*, *a.s.*) is well developed and scoop-shaped. It is bound posteriorly by the fifth annulus, which forms the first complete ring of the body at the anterior end. The circular posterior sucker (Fig. 1*b*, *p.s.*) is centrally attached to the caudal end of the body, which narrows to form a sort of peduncle for its attachment. The diameter of the posterior sucker is approximately half the maximum width of the body.

Coloration. The body (Fig. 1*a*) is profusely pigmented and this pigmentation masks much of the anatomy of this leech. In the living state, the translucent body appears light brown on the dorsal side and against this colour a median longitudinal band (Figs. 1*a*, 2*a*, *m.l.y.b.*) of lemon-yellow colour is visible. The band runs

* This work was started by the writer when he was working as a stipendiary student of the Government of India in the Laboratory of the Zoological Survey of India, Calcutta. As the work was incomplete at that time it was not published. It was, however, continued in the Zoological Laboratory of the University of Lucknow and is now being published from this University with the permission of the late Dr S. L. Hora, formerly Director of the Zoological Survey of India.

† Assistant Professor of Zoology.

‡ Named after the late Dr S. L. Hora, Director, Zoological Survey of India, Calcutta.

from the posterior to the anterior end of the body and it gradually narrows towards its anterior end. On each side of this band are present two series of dark-brown and two of lemon-yellow spots.

Of the two series of dark-brown spots, one is the intermediate series (Fig. 1*a*, *i.db.sp.*), which runs midway between the median longitudinal band and the margin of the body. These spots are merely congregations of dark-brown pigments. Usually there are ten to twelve spots in each series. They lie in the neural or the middle annuli of the complete triannulate somites of the body. The second or the marginal series of dark-brown spots (Fig. 1*a*, *m.db.sp.*) is present at the margin

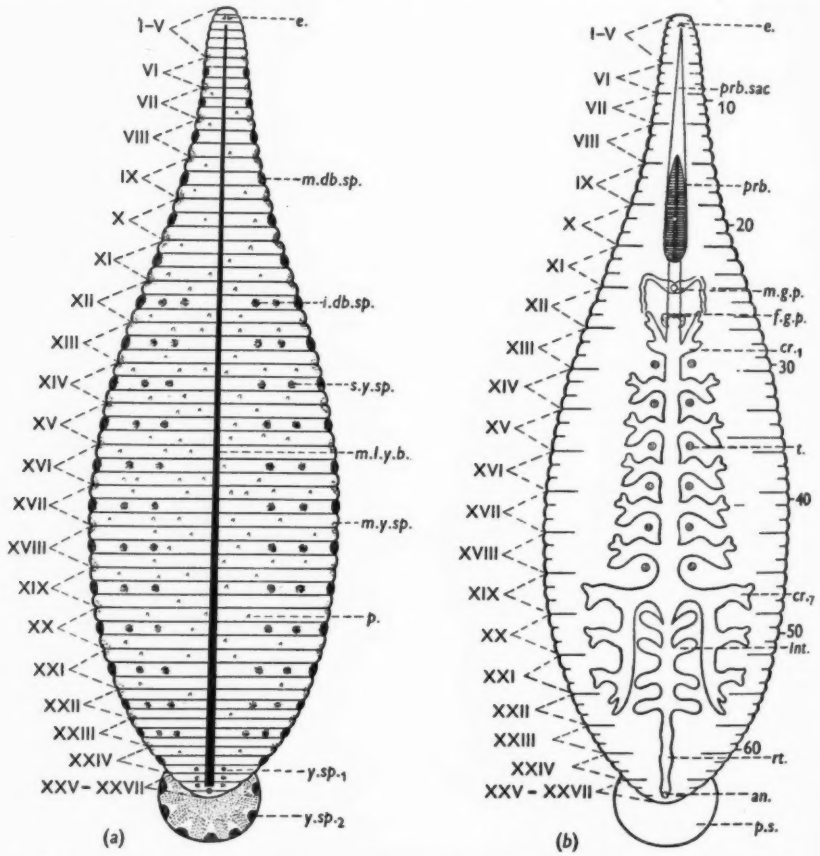


Fig. 1. *Placobdella horai* sp.nov. (*a*) Diagram showing the annulation and metameric spots. (*b*) Diagram showing the alimentary canal and reproductive organs. *an.*, anus; *cr.*$_1$–*cr.*$_7$, seven pairs of crop caeca; *e.*, eyes; *f.g.p.*, female genital pore; *int.*, intestine; *i.db.sp.*, intermediate dark-brown spot; *m.db.sp.*, marginal dark-brown spot; *m.g.p.*, male genital pore; *m.l.y.b.*, median longitudinal yellow band; *m.y.sp.*, marginal yellow spot; *p.*, papillae; *prb.*, proboscis; *prb.sac*, proboscis-sac; *p.s.*, posterior sucker; *rt.*, rectum; *s.y.sp.*, supramarginal yellow spot; *t.*, testis-sac; *y.sp.*$_1$, yellow spots at the broad end of the longitudinal band; *y.sp.*$_2$, yellow spots upon the posterior sucker. Somites numbered in Roman numerals.

of each side of the body. These spots also lie upon the neural annuli and they continue anteriorly almost to the posterior border of the anterior sucker.

Of the two series of lemon-yellow spots, one is supramarginal and the other marginal. The supramarginal spots (Fig. 1*a*, *s.y.sp.*) are present on each half of the body between the two series of dark-brown spots on the neural annuli. The yellow spots of the marginal series (Fig. 1*a*, *m.y.sp.*) are present at the margin of each side of the body between the marginal dark-brown spots. These marginal yellow spots continue for almost the whole length of the body and gradually decrease in size towards the extremities.

In addition to these series of spots, two lateral yellow spots (Fig. 2*a*, *l.y.sp.*) are visible in some specimens at the sides of the cephalic region of the body a little behind the eyes. Three yellow spots are sometimes found on either side at the broad end of the median longitudinal band (Fig. 1*a*, $y.sp._1$) on the 63rd, 64th and 65th annuli. Yellow spots (Fig. 1*a*, $y.sp._2$) are also present on the margin of the posterior sucker and these are separated by radial brown stripes.

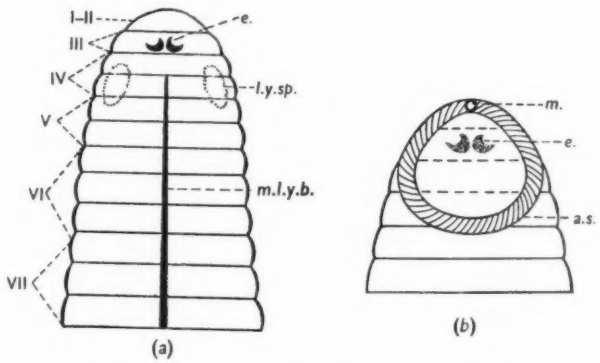


Fig. 2. *Placobdella horai* sp.nov. (*a*) Dorsal view of the anterior end showing the lateral yellow spots at the sides of cephalic region. (*b*) Ventral view of the anterior end showing the anterior sucker and position of mouth. *a.s.*, anterior sucker; *e.*, eyes; *l.y.sp.*, lateral yellow spot; *m.*, mouth; *m.l.y.b.*, median longitudinal yellow band.

Annulation. In most of the specimens, the annulation of the anterior end is rather obscure; but in the remaining part of the body, the annuli are well marked off, and the limits of the complete somites are well defined. The sensillae are embedded within the series of spots seen on the dorsal surface. In specimens which have faded, the sensillae of the intermediate series of spots are clearly visible as minute white diaphanous spots within the pigment masses. By using these regularly distributed dark-brown spots which mask the sensillae, the number of complete somites could be easily counted. Other series of sensillae, however, could not be clearly made out.

The total number of annuli is 66. Usually the 61st and 62nd rings are not well defined and they may appear confluent. In some specimens, the 59th and 60th annuli also appear joined together as a broad ring without a distinct interannular groove.

The annular constituents of somites composing the body of this leech are as follows: the first six annuli constitute the somites I–V, which are all incomplete. The next 57 annuli form nineteen (VI–XXIV) complete triannulate somites, the last three annuli form XXV–XXVII somites, which are all uniannulate.

Digestive system. The oral aperture is small and it is located ventrally at the anterior margin of the anterior sucker (Fig. 2*b*, *m.*). It leads into a large proboscis-sac (Fig. 1*b*, *prb.sac*), which lodges the proboscis. The proboscis-sac extends back to segment XI. The proboscis is rather short as compared with the size of the body. It measures 0·9 mm. in the type specimen, which is 8·4 mm. in length. It communicates behind with a short oesophagus, which is followed by a large crop-stomach occupying the segments XIII–XIX. The crop-stomach has seven pairs of branched caeca (Fig. 1*b*, $cr._1$–$cr._7$), one pair lying in each segment. The first pair of caeca is quite unlike the others; it is directed forwards and extends over three annuli. The succeeding pairs of caeca progressively increase in size and they are directed outwards and slightly backwards. The last (seventh) pair of caeca is the longest and is reflected backwards extending as far as segment XXII. It bears lateral branches. The crop-stomach is followed by the intestine (Fig. 1*b*, *int.*), which lies in the segments XX–XXII between the backwardly directed last pair of the crop caeca. The intestine has four pairs of well-developed lateral caeca, the first two of which are directed forwards and the other two backwards. The rectum (Fig. 1*b*, *rt.*) arises in somite XXIII and terminates in an anal aperture (Fig. 1*b*, *an.*), placed in the last groove between the 65th and 66th annuli.

Generative system. Specimens in the collection are not fully sexually mature.

There are six pairs of testes-sacs (Fig. 1*b*, *t.*). Each is a small round vesicular body placed between the crop caeca of each side. The vas deferens of each side is a narrow tube ending in a short ductus ejaculatorius without any distinct vesicula seminalis; it ultimately leads into the common median prostate chamber or atrium. The latter opens to the exterior by the male genital pore (Fig. 1*b*, *m.g.p.*) located in the groove between the 24th and 25th rings, i.e. between the somites XI and XII.

The ovisacs appear as a pair of small saccular bodies extending hardly over one and a half annuli. They unite anteriorly by their short oviducts and ultimately open to the outside by the female genital pore (Fig. 1*b*, *f.g.p.*) located two annuli behind the male genital aperture in the groove between the second and third annuli of the somite XII, i.e. at XII$a._2/a._3$.

Host and habitat. Over a dozen specimens were collected by the author on 12 December 1948, during the course of a faunistic Survey of Chota Nagpur Division from a lake popularly known as '*Sahib Bandh*' in Purulia, Manbhum District (Bihar). They were all taken from the prawns, *Palaemon lamarrei* H.M. Edw., and were found in every case attached by their posterior suckers to the carapace of the host. In no case more than two specimens were collected from a prawn. The intensity of infection was, however, low; about 5% of the prawns collected were found infected. The fisherman engaged for the collection of the fauna of the lake informed the author that these leeches were very common during the monsoon.

Discussion. The present form conforms to the characters of the genus *Placobdella* R. Blanchard, 1893. So far only five species of this genus have been recorded from India (including Burma and Ceylon), namely *P. emydae* Harding, 1920; *P. ceylanica* (Harding, 1909) Moore, 1924; *P. fulva* Harding, 1924; *P. undulata* Harding, 1924; and *P. inleana* (Oka, 1922) Harding, 1927.

Although the form under consideration differs from these species, it shows some resemblance to *P. fulva* in the dorsal pattern; but it can be easily distinguished from *P. fulva* by the presence of numerous papillae on the dorsal surface of its body and by the position of the gonopores, which are, in *P. horai*, located two annuli further ahead.

Type (no. W3760/1) and paratypes (no. W3761–3763/1) deposited in the collection of the Zoological Survey of India, Indian Museum, Calcutta.

Hemiclepsis bhatiai* sp.nov. (Figs. 3*a*, 3*b*, 4*a*, 4*b*)

External morphology. The body of this leech (Fig. 3*a*, *b*) is claviform. In the type a distinct head is hardly distinguishable, but in some specimens the cephalic region is slightly expanded. The adult specimens are more or less equal in size. The type measures 15·3 mm. in length and 2·3 mm. in width across the broadest part of the body. The anterior sucker (Fig. 4*a*, *o.s.*) is shallow. The posterior sucker is circular and is about two-thirds of the maximum width of the body.

There are three pairs of cup-shaped eyes (Fig. 4*a*, $e._1$–$e._3$), which are arranged in two longitudinal rows on the cephalic region of the body. The eyes of the first pair are the smallest, while those of the second pair are the largest. The eyes of the third pair are intermediate in size between the first and the second pair of eyes. The relative size of the eyes is characteristic of this form. The eyes of the first pair are very closely apposed, while those of the second pair either just touch each other, or are slightly apart. Both the first and the second pair of eyes face antero-laterally. Because the annulation of the anterior part of the body is not very distinct, it is difficult to determine with certainty the annuli on which the first two pairs of eyes are located, but their close proximity indicates that they possibly lie on two consecutive annuli. The eyes of the third pair are always wide apart and they face postero-laterally. In all specimens in the collection they are located one annulus behind the second pair.

Coloration. The colour in the living condition is not recorded by the collectors. The preserved specimens appear light cream-coloured on the dorsal side, against which dull light brown spots are visible. There are fourteen or fifteen spots arranged in a longitudinal series on each half of the dorsal side (Fig. 3*a*) in the intermedial line. These spots constitute the intermediate series. The spots exist on every third ring (sensory or $a._2$) of the body, beginning from the 23rd annulus (somite X), and extend posteriorly to the 67th annulus (somite XXV). Two additional pigment spots (Fig. 3*a*, *p.s.*), which are paramedian in position, are present on the 67th annulus (XXV $a._1$ $a._2$). The type specimen shows a few supramarginal spots on the right side, but they are not seen in other specimens.

* Named after Dr M. L. Bhatia, of the Department of Zoology, University of Delhi.

Mounted specimens further reveal that there are minute yellowish brown pigment granules which are densely deposited on the dorsal side of each annulus. The inter-annular grooves are devoid of these pigments. The chromatophores are present on the dorsal side in the mid-longitudinal field between the two series of intermediate spots. They are arranged in irregularly distributed groups. Several supramarginal spots are present on the dorsal aspect of the posterior sucker (Fig. 3*a*, *s.sp.*).

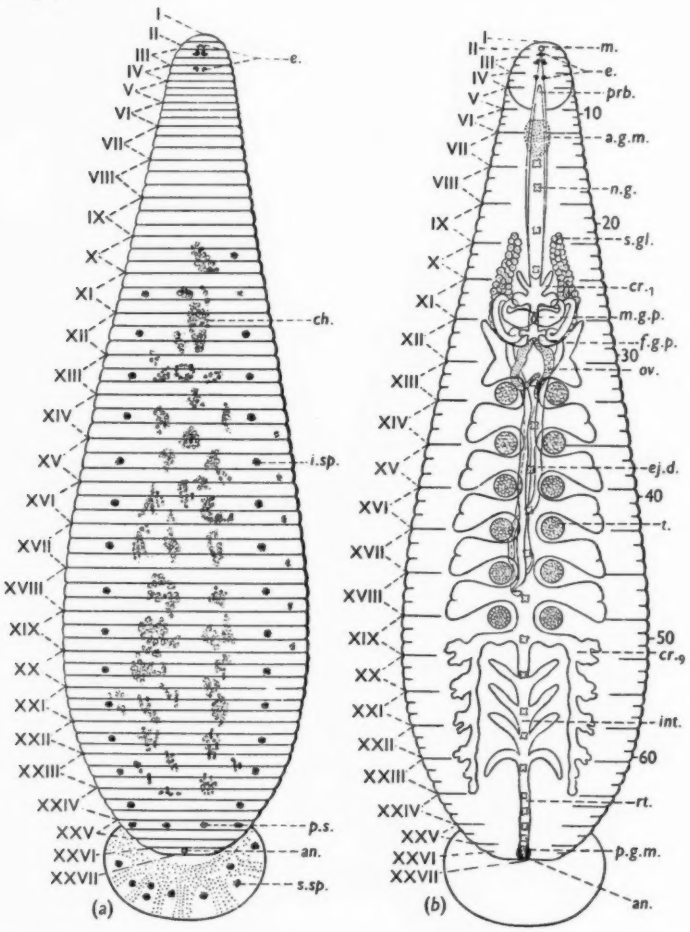


Fig. 3. *Hemiclepsis bhatiai* sp.nov. (*a*) Diagram showing the annulation and pigment spots. (*b*) Diagram showing the alimentary canal and reproductive organs. *a.g.m.*, anterior ganglionic mass; *an.*, anus; *ch.*, chromatophores; *cr.*$_1$–*cr.*$_9$, nine pairs of crop caeca; *e.*, eyes; *ej.d.*, ejaculatory duct; *f.g.p.*, female genital pore; *int.*, intestine; *i.sp.*, intermediate series of spots; *m.*, mouth; *m.g.p.*, male genital pore; *n.g.*, nerve ganglion; *ov.*, ovisacs; *t*, testis-sac; *prb.*, proboscis; *p.g.m.*, posterior ganglionic mass; *rt.*, rectum; *s.gl.*, salivary gland; *p.s.*, paramedian spots at the posterior end of the body; *s.sp.*, supramarginal spots on the posterior sucker; Somites numbered in Roman numerals.

Annulation. There are seventy annuli (Fig. 3*a*), which constitute twenty-seven somites as follows:

Somites I and II are uniannulate, III and IV are biannulate. The second annulus of the somite III is quite distinct, but the annuli in front of this ring are obscure. The number of annuli of this part has been determined on the basis of the position of the first and the second pair of eyes. The somites V–XXIV, both inclusive, are triannulate and complete. The somite XXV is also biannulate and the last two somites, XXVI and XXVII, are uniannulate.

The first nine annuli are represented only by the dorsal halves, the ventral halves of these annuli are absorbed by the anterior sucker. The tenth annulus is the first complete ring of the body.

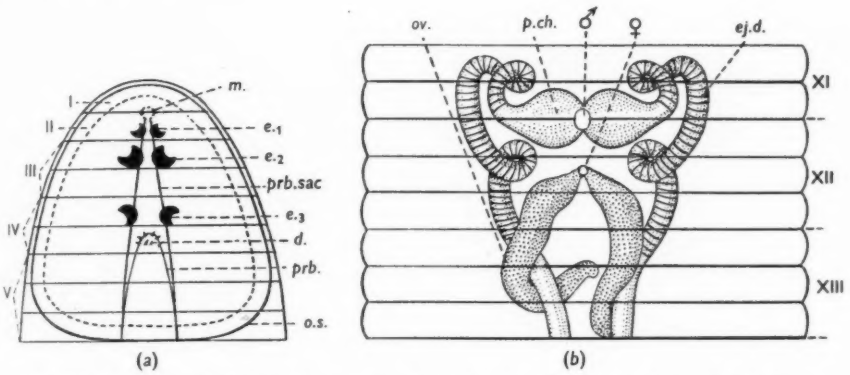


Fig. 4. *Hemiclepsis bhatiai* sp.nov. (*a*) Dorsal view of the anterior end showing the position of the mouth and eyes, and the denticles on the proboscis. (*b*) Diagram showing the terminal parts of the ejaculatory canals and ovisacs. *d.*, denticles; $e._1$–$e._3$, three pairs of eyes; *ej.d.*, ejaculatory duct; *m.*, mouth; *ov.*, ovisac; *o.s.*, anterior sucker; *prb.*, proboscis; *p.ch.*, prostate chamber; *prb.sac*, proboscis-sac. Somites numbered in Roman numerals.

Digestive system. The oral aperture (Fig. 4*a*, *m.*) is small, and is located within the anterior sucker, a little in front of the first pair of eyes. It leads into the proboscis-sac (Fig. 3*b*, *prb.sac*), which extends as far as segment X and accommodates the proboscis. The proboscis measures 3·4 mm. in length and shows fine denticles (Fig. 4*a*, *d.*) at its anterior end. Moore (1938*a*) also recorded the presence of denticles at the tip of the proboscis in a specimen of *Helobdella punctato-lineata* from Puerto Rico. The proboscis opens into the crop-stomach, which runs from the segment XI to XIX. The crop-stomach has nine pairs of caeca (Fig. 3*b*, $cr._1$–$cr._9$) of which the first two pairs are situated in front of the gonopores. The first and the second pair of caeca are small and are so close together that they give the appearance of a single pair of caeca with a pair of horn-like anterior prolongations. The third pair of caeca is much bigger than the first two pairs and is quite unlike the succeeding pairs of caeca. A pair of very small lateral inflations (probably oesophageal organs) of the crop-stomach is also distinguishable in the region between the second and the third pair of caeca. The next five pairs of caeca are of the usual type and are lobed.

They gradually increase in size from before backwards and are directed outwards and slightly backwards. The last pair of caeca is long and is reflected backwards, extending as far as segment XXIII. It bears secondary lateral branches which gradually decrease in size towards the posterior end of the body. The intestine (Fig. 3*b*, *int.*) arises from the crop-stomach in the segment XX and continues as far as segment XXIII. The intestine, like that of all other Glossiphonid leeches, bears four pairs of caeca, the first three pairs being directed forwards, while the fourth pair is directed backwards. The intestine continues into a short rectum, which terminates in the anal aperture (Fig. 3*b*, *an.*) located on the last annulus.

There is a pair of compact salivary glands (Fig. 3*b*, *s.gl.*), one on either side, near the base of the proboscis. Each gland is elongated and is composed of large secreting cells.

Generative system. There are six pairs of testes-sacs (Fig. 3*b*, *t.*) alternating with the crop caeca of each side. The first pair lies in front of the fourth pair of crop caeca at XIII/XIV. The vasa efferentia from the testes-sacs of each side open into a narrow vas deferens, which is continued into a long ductus ejaculatorius (Figs. 3*b*, 4*b*, *ej.d.*). The terminal part of each ejaculatory duct forms loops. The circular muscle fibres of the terminal part of the ejaculatory ducts appear prominent. A distinct vesicula seminalis is not distinguishable. Each ductus ejaculatorius ends in a large pear-shaped atrial cornus, which meets its fellow of the opposite side in the mid-longitudinal line to form a common prostate chamber (Fig. 4*b*, *p.ch.*) or atrium. The prostate chamber eventually opens to the exterior by a prominent oval aperture (Fig. 3*b*, *m.g.p.*, Fig. 4*b*, ♂) upon the groove between the somites XI and XII.

The ovisacs (Figs. 3*b*, 4*b*, *ov.*) are a pair of elongated saccular bodies, which extend back over four or five annuli. Both meet anteriorly and finally open to the exterior by the female genital pore (Fig. 3*b*, *f.g.p.*, Fig. 4*b*, ♀) situated in the middle of the annulus XII$a._2$, i.e. one and a half annuli behind the male pore.

Host and habitat. The specimens were collected on 20 November 1947 by Dr H. C. Ray and Mr K. S. Pradhan of the Zoological Survey of India from a rocky pool 'Sitkundi' in Kalipahar, about 7 miles south-west of Monghyr District (Bihar), during the course of a survey of the Kharagpur hills. They were not associated with any host, but were at rest under stones, to which they were found attached.

Discussion. The form under consideration resembles the genus *Hemiclepsis* Vejdovsky, 1883, as well as the genus *Paraclepsis* Harding, 1924, in having more than seven pairs of crop caeca, so the number of crop caeca does not help in solving the difficulty of assigning the present form to either of these two genera. The position of the mouth aperture is an important taxonomic feature. In *Hemiclepsis* the mouth is located within the anterior sucker, whereas in *Paraclepsis* it is subterminal and leaves the anterior sucker imperforate. In the present form, the position of the mouth aperture within the oral sucker, which it perforates near the anterior rim, is a decisive factor in determining the position of this form in the genus *Hemiclepsis*.

So far as the author knows, only two species of the genus *Hemiclepsis* are at present

known from India, namely, *H. marginata* Müller, 1774 and *H. viridis* Chelladurai, 1934.

The present form, while it differs markedly from *H. marginata*, resembles *H. viridis* in the number of its eyes, but it can be distinguished from *H. viridis* by the absence of papillae on its body, by the size of its second pair of eyes, which are the largest, and lastly by the position of its gonopores.

Type (no. W 3764/1) and paratypes (no. 3765–3766/1) deposited in the collection of the Zoological Survey of India, Indian Museum, Calcutta.

Key to the Indian species of the genus Hemiclepsis *Vejdovsky*, 1883

I. Eyes two pairs. **H. marginata** Müller, 1774
Eyes three pairs. II

II. Annuli 70, papillae absent, second pair of eyes largest, gonopores one and a half annuli apart, metameric pigment spots present, male pore at 27/28, female pore on 29. **H. bhatiai** sp.nov.

Annuli 71, papillae present, third pair of eyes largest, gonopores two annuli apart, metameric pigment spots absent, male pore at 28/29, female pore at 30/31. **H. viridis** Chelladurai, 1934

Batracobdella hardingi* sp.nov. (Figs. 5*a*, 5*b*)

External morphology. A single specimen of this species is present in the collection. The body is short, broad and oval. The length is 3·0 mm., while the maximum width across the broadest part of the body is 1·5 mm.

Three pairs of cup-shaped eyes (Fig. 5, $e._1$–$e._3$) are present. The eyes of the first pair† are very small and may easily escape detection. They are eclipsed by the large second pair of eyes, with which they are closely associated, and each lies beneath the inner half of the large eye of its own side facing anteriorly. The eyes of the second pair are the largest. They are located on the third annulus, but extend forwards into the second annulus, and are directed antero-laterally. The third pair of eyes is situated on the fifth annulus. They are intermediate in size between the first and the second pair of eyes. The eyes of the third pair face outwards and backwards.

The anterior sucker is deep and has absorbed the ventral halves of the first six annuli of the body. It has a well-defined rim all round. The ventral halves of the seventh and eighth annuli ($Va._1$, $a._2$) coalesce to form the posterior rim of the anterior sucker. The ninth annulus ($Va._3$) is the first complete ring of the body. The posterior sucker (Fig. 5, *p.s.*) is less than half the maximum width of the body.

Coloration. The colour in life is not recorded. The preserved specimen also does not show any pigment. The dorsal surface presents faint longitudinal stripes, presumably due to the bands of longitudinal muscle fibres.

* Named after Mr W. A. Harding, the author of Rhynchobdellae in Hirudinea, *Fauna of British India*.

† This pair of eyes can only be seen under the microscope by carefully changing the focus. The peculiar position of these eyes seems to be normal, because all the anterior annuli are quite distinct and are not overlapping.

Receptors. The receptors are present in large numbers all over the dorsal surface of the body, and for this reason the dorsal surface appears areolated. The receptors are thickly set and form irregular rows on each annulus. Some of the receptors appear circular, others transversely oval.

Annulation. The annuli are all distinct. The total number of annuli is 70. They are divisible into twenty-seven somites as follows:

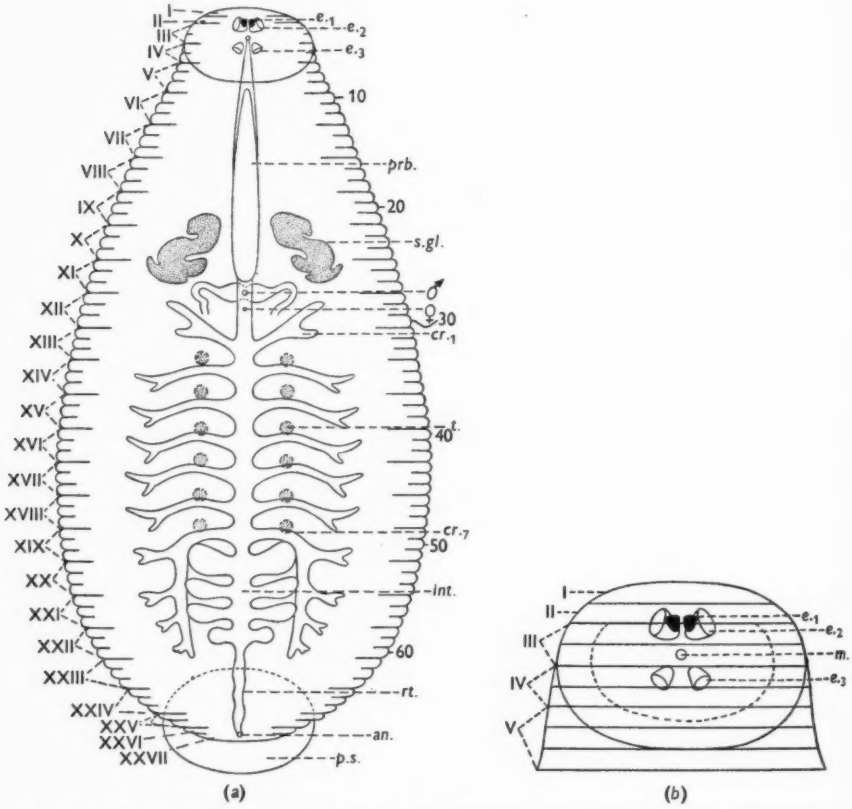


Fig. 5. *Batracobdella hardingi* sp.nov. (*a*) Diagram showing the alimentary canal and reproductive organs. *an.*, anus; *cr.*$_1$–*cr.*$_7$, seven pairs of crop caeca; *e.*$_1$–*e.*$_3$, three pairs of eyes; *int.*, intestine; *prb.*, proboscis; *p.s.*, posterior sucker; *rt.*, rectum; *s.gl.*, salivary glands; *t.*, testis-sac. Somites numbered in Roman numerals. (*b*) Dorsal view of the anterior end showing the position of the eyes and mouth. *e.*$_1$–*e.*$_3$, three pairs of eyes; *m.*, mouth.

The first and the second somites are uniannulate, the third and the fourth are biannulate. The segments V–XXIV are all triannulate and are the complete somites of the body. The somite XXV is biannulate. The last two somites, XXVI and XXVII, are uniannulate.

Digestive system. The oral aperture (Fig. 5, *m.*) is small and is located (Figs. 5*a*, *b*) near the centre of the anterior sucker, between the second and the third pair of

eyes. It leads into the proboscis-sac which lodges the proboscis (Fig. 5*a*, *prb.*) that extends back into segment XI. The proboscis opens into the crop-stomach, which occupies the segments from XII to XIX. The crop-stomach has seven pairs of bifid lateral caeca (Fig. 5*a*, $cr._1$–$cr._7$). The first pair of caeca is directed forwards and outwards. The last pair of caeca is long and reflected backwards; it extends to the end of the segment XXII and bears lateral branches which bifurcate at their tips. The crop-stomach leads into the intestine (Fig. 5*a*, *int.*), which extends from the segment XIX to XXIII. The intestine has four pairs of lateral caeca, which lie between the last pair of crop caeca. The first pair of caeca is the largest, the next two are slightly smaller while the fourth pair is the smallest. The intestine narrows into a short rectum (Fig. 5*a*, *rt.*) which opens to the outside by an anal aperture (Fig. 5*a*, *an.*), located in the groove between the last two annuli of the body.

A pair of compact salivary glands (Fig. 5*a*, *s.gl.*) lies at the base of the proboscis and the ducts from these glands open into the base of proboscis.

Generative system. Details of this system could not be made out from the single specimen available for study.

There are six pairs of small round testes-sacs (Fig. 5*a*, *t.*), which alternate their position with the crop caeca of each side. The first pair of testes-sacs is placed in front of the second pair of crop caeca. The terminal part of each ductus ejaculatorius enters a small common prostate chamber, which opens to the exterior by the male genital pore situated in the groove between the segments XI and XII.

The ovisacs could not be detected in the specimen. The female genital pore is located on the middle of the annlus XII$a._2$.

Host and habitat. The collection was made on 20 November 1947 by Dr H. C. Ray and Mr K. S. Pradhan of the Zoological Survey of India from a rocky pool 'Sitkundi' in Kalipahar about 7 miles south-west of Monghyr District (Bihar), during the course of a survey of the Kharagpur hills. The specimens were taken from under the stones to which they were found attached.

Discussion. The number of eyes and that of the crop caeca, and the position of the oral aperture within the anterior sucker are the features which include this form within the genus *Batracobdella* Viguier, 1879. Only two species of this genus are at present known from India, namely, *B. reticulata* (Kaburaki, 1921*b*), Autrum, 1936, and *B. lobata* (Bhatia, 1934), Autrum, 1936. The present form differs from these species in the number of eyes and annuli. In the number of eyes, however, it resembles the foreign species *B. cancricola* (Oka, 1928), Autrum, 1936, but differs from it in other respects. It is interesting to note that in the arrangement of the eyes this species somewhat resembles the species *B. tricarinata* (Blanchard, 1897), in which, as Moore (1938) states, the first pair of small eyes is situated between the second pair of large eyes, and also *Glossiphonia annanalei* Oka, 1922, another Indian Glossiphonid leech.

Type (no. W3767/1) deposited in the collection of the Zoological Survey of India, Indian Museum, Calcutta.

Key to the Indian species of the genus Batracobdella *Viguier*, 1879

I. Eyes three pairs, annuli 70. **B. hardingi** sp.nov.
Eyes two pairs, annuli 72. II

II. Papillae absent, male pore at 24/25, female pore at 26/27, crop caeca entire.
B. lobata (Bhatia, 1934), Autrum, 1936
Papillae present, male pore at 26/27, female pore at 28/29, crop caeca branched.
B. reticulata (Kaburaki, 1921*b*), Autrum, 1936

Glossiphonia heteroclita (Lin. 1761) (Fig. 6)

External morphology. About a dozen specimens of this species are present in the collection. The specimens are more or less equal in size measuring 9·0–10·5 mm. in length and about 2·5 mm. in maximum width. The body is ovate-acuminate and dorso-ventrally flattened. The posterior sucker is circular and is less than half of the maximum width of the body.

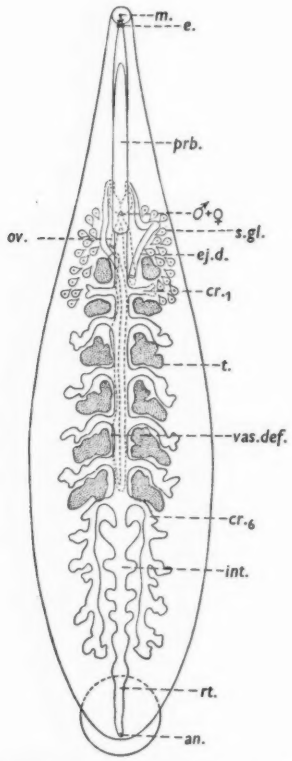


Fig. 6. *Glossiphonia heteroclita* (Lin.). Diagram showing the alimentary canal and reproductive organs (for the sake of clarity, certain structures are shown only on one side). *an.*, anus; *cr.*$_1$–*cr.*$_6$, six pairs of crop caeca; *e.*, eyes; *ej.d.*, ejaculatory duct; *int.*, intestine; *m.*, mouth; *ov.*, ovisacs; *prb.*, proboscis; *rt.*, rectum; *s.gl.*, salivary glands; *t.*, testes-sac, *vas.def.*, vas deferens.

Three pairs of eyes are located on consecutive annuli. The eyes of the first pair are the smallest and are closely apposed. The eyes of the second pair are wide apart and are larger than those of the first pair. Both the first and the second pair of eyes face obliquely forwards. The eyes of the third pair are slightly larger than those of the second pair, and they are placed just behind the latter facing postero-laterally. In a typical case, the eyes of the second and the third pair on each side are closely pressed against each other and thus the eyes show a triangular arrangement.

Coloration. The specimens preserved in alcohol have lost their original colour and appear whitish. Yellowish pigment is, however, present on the dorsal surface of the body. Careful examination of freshly mounted specimens further reveals three fine transverse stripes of dark pigment across each annulus. These stripes are, however, not regularly seen on all the annuli of the body, so the total number of annuli cannot be counted.

Annulation. The annuli of the body are indistinct and the total number of annuli could not be determined. At places, they could be distinguished by the annular stripes just mentioned.

Digestive system. The mouth (Fig. 6, *m.*) is situated in the centre of the anterior sucker. It leads into the proboscis-sac which extends posteriorly beyond the gonopore. The proboscis (Fig. 6, *prb.*) is well developed and it measures 2·3 mm. in a specimen of 10·5 mm. in length. It opens into the crop-stomach which is thin-walled (Fig. 6) and has six pairs of caeca (Fig. 6, $cr._1$–$cr._7$). The caeca progressively increase in size posteriorly and appear branched at their ends. In specimens gorged with blood, the caeca present a notched appearance. Kaburaki (1921*b*) refers to this feature of the crop as characteristic of this species. The last pair of crop caeca is long and it is reflected backwards, extending beyond the last pair of intestinal caeca. It bears secondary branches. The crop-stomach is continued into the intestine (Fig. 6, *int.*), which shows columnar epithelial cells in its wall. These cells of the intestinal wall are very prominent in all the specimens in the collection and they sharply demarcate the intestinal region from the crop-stomach. The intestine has four pairs of intestinal caeca, which lie between the last pair of the crop caeca. The intestinal caeca gradually decrease in size towards the posterior end of the body; the first pair of these caeca is, however, considerably larger than the second pair. They are all directed antero-laterally. The intestine passes into the rectum (Fig. 6, *rt.*), which opens to the outside by the anal aperture (Fig. 6, *an.*), located in the groove between the last two annuli of the body.

Large secreting cells of the salivary glands (Fig. 6, *s.gl.*) are diffused over a number of annuli on either side of the body near the anterior end of the crop-stomach.

Generative system. There are six pairs of large testes-sacs (Fig. 6, *t.*), which lie between the crop caeca of each side. They are variable and irregular in shape. The vas deferens (Fig. 6, *vas.def.*) of each side, which receives the efferent ducts from the testes-sacs of its own side, extends as far back as the last pair of testes-sacs. It is then continued into a long ductus ejaculatorius (Fig. 6, *ej.d.*), which has a much wider lumen and runs forward. In most specimens, the vas deferens and

ductus ejaculatorius of each side are, however, tortuously coiled near the first pair of crop caeca. A distinct vesicula seminalis is not distinguishable. Each ejaculatory duct contains sperms densely crowded within it. The terminal part of each ejaculatory duct runs by the side of the base of proboscis, where it sharply bends downwards and backwards to enter into the prostate chamber (atrium), which opens to the exterior by the common genital pore (Fig. 6, ♂+♀).

The ovisacs (Fig. 6, *ov.*) are a pair of elongated tubes which meet each other anteriorly and open into the common gonopore.

Host and habitat. The specimens were collected, together with *Hemiclepsis bhatiai* sp.nov. and *Batracobdella hardingi* sp.nov., by Dr H. C. Ray and Mr K. S. Pradhan of the Zoological Survey of India on 20 November 1947 from a rocky pool 'Sitkundi' in Kalipahar situated about seven miles south-west of Monghyr District (Bihar), during the course of a survey of the Kharagpur hills. They were taken from under the stones.

Remarks. Harding (1920) referred a number of specimens collected from Chilka Lake, Orissa, to the species *Glossiphonia heteroclita*. Subsequently, Kaburaki (1921*a*), working on some additional material from Chilka Lake, found that the form identified by Harding (1920) as *G. heteroclita* was apparently *G. ceylanica* Harding, 1909. Later Moore (1924), however, showed that *G. ceylanica* Harding, 1909, is really *Placobdella ceylanica* (Harding, 1909). Consequently in the *Fauna of British India*, the species *G. heteroclita* is reported to have been recorded from Burma (Oka, 1922). In view of this fact, the present find seems to be the first record of this species in India.

Key to the Indian species of the genus Glossiphonia *Johnson*, 1816*

I. Testes ten pairs. **G. complanata** (Lin. 1758)
Testes six pairs. II

II. Eyes typically arranged in three groups corresponding to the angles o an equilateral triangle, gonopore common. III
Eyes typically arranged in tandem, gonopores separate. IV

III. Papillae and pigment spots present, crop caeca entire. **G. weberi** Blanchard, 1897
Papillae and pigment spots absent, crop caeca slightly branched or tending to branch at the end. **G. heteroclita** (Lin. 1761)

IV. Annuli 68, first pair of eyes between the second pair, male pore at 24/25, female pore at 26/27. **G. annandalei** Oka, 1922
Annuli 70, first pair of eyes in front of the second pair, male pore at 26/27, female pore at 28/29. **G. cruciata** Bhatia, 1930*a*

I wish to express the debt of gratitude which I owe to Professor J. P. Moore of the University of Pennsylvania, Philadelphia, U.S.A., for the trouble he has taken in critically examining the manuscript of this paper and also for giving valuable suggestions. I wish to thank Professor M. B. Lal of the University of Lucknow, India, for the kind interest he has taken in this work, and also the late Dr S. L.

* It also includes the species recorded in the *Fauna of British India* from Burma and Ceylon. It excludes the species *Glossiphonia reticulata* Kaburaki, which has been rightly placed under the genus *Batracobdella* Viguier by Autrum (1936).

Hora, Director, Zoological Survey of India, Calcutta, for all the facilities he gave me when the work was being carried out in the Laboratory of the Zoological Survey of India.

BIBLIOGRAPHY

AUTRUM, H. (1936). Hinudineen. *Bronns Klassen und Ordnungen des Tierreichs*, Teil 1, Lief 1, 1–95.

BHATIA, M. L. (1930*a*). Sur une nouvelle hirudinee rhynchobdelle, *Glossiphonia cruciata* n.sp., provenant du vivier à truites d'Achha Bal, Kashmir. *Ann. Parasit. hum. comp.* **8**, 344–8.

BHATIA, M. L. (1930*b*). On the anatomical details of *Placobdella emydae* Harding. A leech parasitic on Indian turtles. *Zool. Anz.* **91**, 225–43.

BHATIA, M. L. (1934). Nouvelle sangsue rhynchobdellide *Glossiphonia lobata* n.sp., de l'établissement de pisciculture d'Achha Bal, Kashmir. *Ann. Parasit. hum. comp.* **12**, 121–9.

BHATIA, M. L. (1939). On some leeches from Kashmir. *Bull. Punj. Univ. Zool.* **2**, 1–17.

CASTLE, W. E. (1900). The metamerism of the Hirudinea. *Proc. Amer. Acad. Arts Sci.* **35**, 285–303.

CHELLADURAI, J. E. (1934). On a new Indian leech, *Hemiclepsis viridis* sp.nov. *Rec. Indian Mus.* **36**, 345–52.

HARDING, W. A. (1910). A revision of the British leeches. *Parasitology*, **3**, 130–201.

HARDING, W. A. (1920). Fauna of Chilka Lake. Hirudinea. *Mem. Indian Mus.* **5**, 509–17.

HARDING, W. A. (1924). Descriptions of some new leeches from India, Burma and Ceylon. *Ann. Mag. Nat. Hist., Lond.*, **14** (9), 489–99.

HARDING, W. A. & MOORE, J. P. (1927). *Fauna of British India. Hirudinea.* London, 1–302.

KABURAKI, T. (1921*a*). On some leeches from the Chilka Lake. *Mem. Indian Mus.* **5**, 661–75.

KABURAKI, T. (1921*b*). Notes on some leeches in the collection of Indian Museum. *Rec. Indian Mus.* **22**, 689–719.

KHAN, H. (1944). Study in diseases of fish. Infestation of fish with leeches and fish lice. *Proc. Indian Acad. Sci.* **19** B, 171–5.

MANN, K. H. (1953*a*). The segmentation of leeches. *Biol. Rev.* **28**, 1–15.

MANN, K. H. (1953*b*). A revision of the British leeches of the family Glossiphoniidae with a description of *Batracobdella paludosa* (Carena, 1824), a leech new to the British fauna. *Proc. Zool. Soc., Lond.*, **123**, 377–91.

MOORE, J. P. (1900). A description of *Microbdella biannulata*, with especial regard to the constitution of the leech somites. *Proc. Acad. Nat. Sci. Philad.* **5**, 50–73.

MOORE, J. P. (1924). Notes on some Asiatic leeches (Hirudinea) principally from China, Kashmir and British India. *Proc. Acad. Nat. Sci. Philad.* **76**, 343–88.

MOORE, J. P. (1930). Leeches from China with descriptions of new species. *Proc. Acad. Nat. Sci. Philad.* **82**, 169–192.

MOORE J. P. (1938*a*). *Helobdella punctata-lineata*, a new leech from Puerto Rico. *Puerto Rico J. Pub.. Hlth*, **14**, 422–9.

MOORE, J. P. (1938*b*). Additions to our knowledge of African leeches. *Proc. Acad. Nat. Sci. Philad.* **90**, 297–360.

OKA, A. (1910). Synopsis der Japanischen Hirudineen, mit diagnosen der neuen species. *Annot. Zool. Jap., Tokyo*, **7**, 165–83.

OKA, A. (1922). Hirudinea from Inle Lake, S. Shan States, Burma. *Rec. Indian Mus.* **24**, 521–534.

RAJ, P. J. S. (1951). On a new species of *Ozobranchus* (Annelida: Hirudinea: family Ichthyobdellidae) from Porto Nova, S. India. *J. Zool. Soc. India*, **3**, 1–5.

RAJ, P. J. S. (1954*a*). On a new species of marine leech of the genus *Branchellion* (family Ichthyobdellidae), from the Indian coast. *Rec. Indian Mus.* **52**, 249–56.

RAJ, P. J. S. (1954*b*). A synopsis of the genus *Ozobranchus* (de Quatrefages, 1852) (*Hirudinea, Annelida*). *J. Bom. Nat. Hist. Soc.*, **52**, 473–480.

WHITMAN, C. O. (1890). Spermatophores as a means of hypodermic impregnation. *J. Morph.* **4**, 361–406.

Parasitology (1960), **50**, 303–322
With 40 text-figures
Printed in Great Britain

Some observations on *Parabothrium gadi-pollachii* (Rudolphi, 1810) and *Abothrium gadi* van Beneden 1870 (Cestoda: Pseudophyllidea) including an account of their mode of attachment and of variation in the two species

By H. HARFORD WILLIAMS, Ph.D.
Department of Zoology, University College of Wales, Cardiff

(*Received* 28 *July* 1959)

The order Pseudophyllidea includes about forty genera and well over a hundred species but many of the specific descriptions are incomplete. Numerous helminthologists, among them Cooper (1918), Nybelin (1922), Stunkard (1948), Wardle & McLeod (1952) and Rausch (1954–6), have discussed the uncertainty and confusion regarding the taxonomy, anatomy and relationships of many cestodes belonging to the order. Two interesting members of the Pseudophyllidea, namely, *Parabothrium gadi-pollachii* (Rudolphi, 1810) and *Abothrium gadi* Beneden, 1870, are often found in some marine fishes belonging to the Gadidae. The separation of these two worms presents some difficulties because of the similarity in their external appearance. Neither has a true scolex and both appear to overlap in their host-distribution. Dollfus (1953) has already summarized our knowledge of *P. gadi-pollachii* and *A. gadi* and included an account of the taxonomy together with a list of synonyms for these two species. Cooper (1918) described *A. gadi* as *A. rugosum* (Batsch, 1786) but there is an indication in the description and figures that some of his material may have been *P. gadi-pollachii*. The latter species and *A. gadi* were described briefly by Nybelin (1922), who concentrated on describing the genitalia, while Woodland (1927) gave an account of immature specimens of *P. gadi-pollachii*. As previous descriptions have been incomplete it was decided to re-investigate *P. gadi-pollachii* and *A. gadi*. The descriptions have been made on a comparative basis in order to emphasize the differences and similarities of their structure.

Before describing the anatomy of the two species an account will be given here of their occurrence in the host fishes examined during the investigation.

THE OCCURRENCE OF *PARABOTHRIUM GADI-POLLACHII* AND *ABOTHRIUM GADI* IN SOME GADIDAE

According to Dollfus (1953), the hosts in which both *P. gadi-pollachii* and *A. gadi* are usually found are *Gadus pollachius* (L) (pollack), *G. aeglifinus* L. (haddock), *G. callarias* L. (cod), *G. virens* L. (coalfish) and *Merluccius merluccius* (L) (hake). He has mentioned the geographical distribution of the two cestodes and recorded their abnormal occurrence in hosts other than the above-named. During the present investigation *P. gadi-pollachii* and *A. gadi* were found in the

cod and the pollack, while *A. gadi* was found also in the haddock. Table 1 includes data concerning the degree of infestation of the fishes with these two worms and the locality and depth from which the fishes were obtained. It is possible that *P. gadi-pollachii* is more specific to the pollack than was hitherto believed, as in over a hundred other individual fishes, including five species belonging to the Gadidae, only one was found and that in the cod. Neither *P. gadi-pollachii* nor *A. gadi* was found in the coalfish and hake although many specimens were examined. The anterior extremity of *P. gadi-pollachii* was firmly fixed within the pyloric caecum of the host, the remainder of the strobila lying in the lumen of the intestine closely applied to the mucosa. Usually one worm occupied a caecum but in three cases two were found side by side. On one occasion a specimen was found in the intestine not very far from the caeca, its anterior end being firmly embedded in and fixed to the intestinal wall (Fig. 17). The anterior end in *A. gadi* also was usually fixed, one or two together within a caecum, the remainder of

Table 1. *The occurrence of* Parabothrium gadi-pollachii *and* Abothrium gadi *in the fishes examined*

Host ...	*Gadus pollachius* (L) (pollack)	*G. callarias* L. (cod)	*G. aeglifinus* L. (haddock)
No. examined	7	20	19
No. parasitized	3	4	2
Parasite	*Parabothrium gadi-pollachii* (15 specimens) *Abothrium gadi* (1 specimen)	*Parabothrium gadi-pollachii* (1 specimen) *Abothrium gadi* (9 specimens)	*Abothrium gadi* (5 specimens)
Habitat	Intestine and caeca	Intestine and caeca	Intestine and caeca
Depth	95 fathoms	65–200 fathoms	65–108 fathoms
Locality	North-east Atlantic	North-east Atlantic	North-east Atlantic
Months of year	July	July and September	July and September

the strobila being in the lumen of the intestine closely applied to the mucosa and sometimes very weakly adhered to it. One large specimen from the cod, however, was found free in the posterior half of the intestine and one small specimen from the haddock was near the caeca, its anterior extremity being embedded in and fixed to the intestinal wall (Fig. 18) as in one of the *P. gadi-pollachii*. It is interesting to note that Woodland (1927) recorded three specimens of *P. gadi-pollachii* from the intestine of the pollack in one of which the anterior end had penetrated the gut wall and consequently protruded into the coelom. He found another specimen free in the coelomic cavity of a different individual of the same host species.

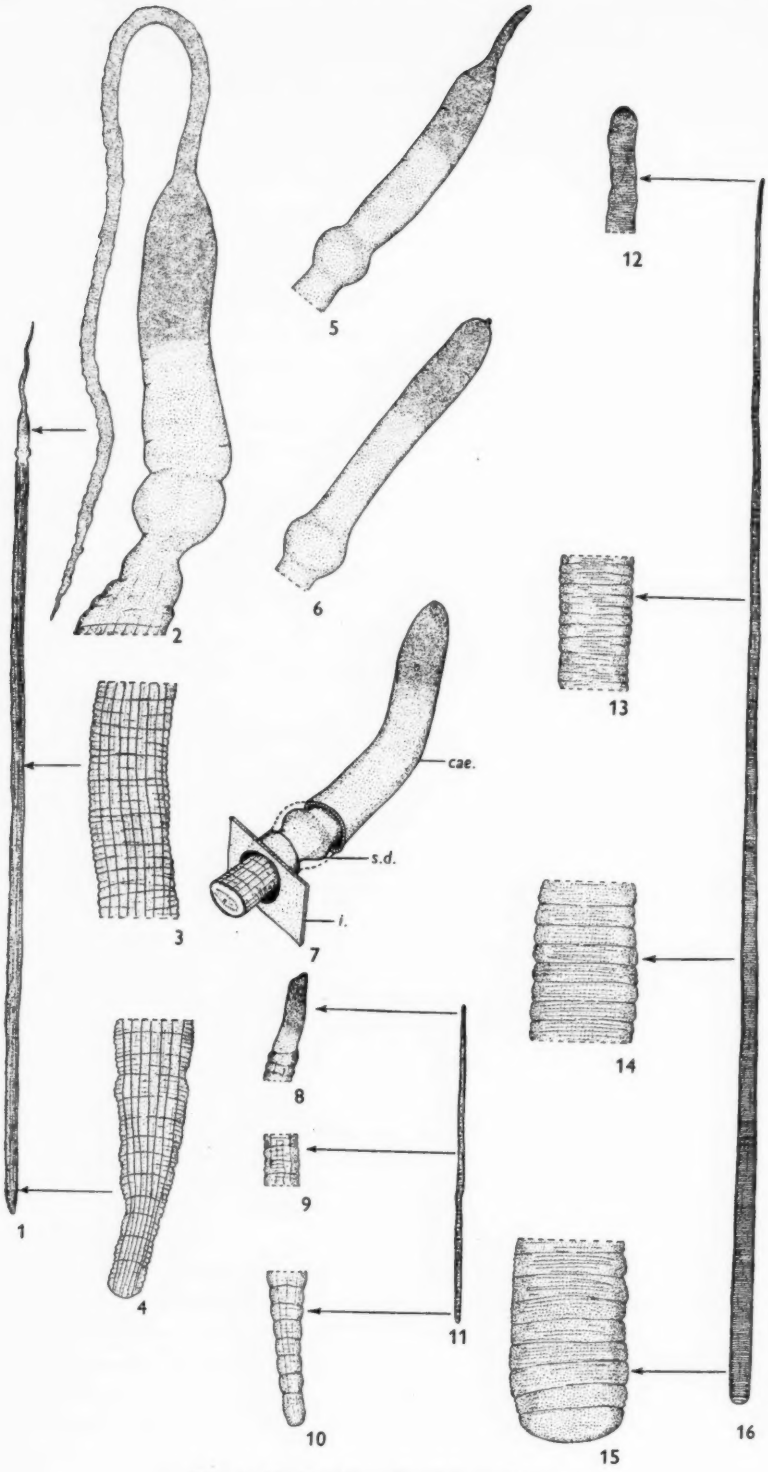
THE ANATOMY OF *PARABOTHRIUM GADI-POLLACHII* AND *ABOTHRIUM GADI*

In order to investigate the anatomy of *P. gadi-pollachii* and *A. gadi* whole mounts and serial sections in three planes of both species were made. For whole mounts satisfactory results were obtaind only when the cuticle and superficial

musculature of the body, as far as the deep longitudinal muscles, were separated from the rest of the strobila, a method used by Markowski (1949) for a study of species of the genus *Diphyllobothrium*. A full account of this method was given by Heyneman (1959). The diazo-staining technique of Smyth & Johri (1956) has given good results for whole mounts, while serial sections were stained with Heidenhain's Azan or Ehrlich's haematoxylin and Orange G or eosin.

External features. (*a*) *The strobila.* The specimens of *P. gadi-pollachii* and *A. gadi* from the pollack were white in colour while those from the cod were yellowish white. Specimens of *A. gadi* from the haddock were also yellowish white. This slight variation in colour of the parasites may be due to differences in the feeding habits of the hosts. According to Brown & Cheng (1946), the cod and haddock consume large numbers of crustaceans, and it is in these two fishes that the yellow colour of the parasites is more apparent, while the pollack, according to Jenkins (1950), feeds mainly on fishes.

The length of the almost-parallel-sided strobila of *P. gadi-pollachii* ranged from 105 to 300 mm.; the breadth measured 2–3 mm. throughout most of the length, but the last 10–25 mm. tapers to a minimum breadth of 1 mm. (Figs. 1–4). Specimens of *A. gadi*, on the other hand, were not parallel-sided, but showed a distinct but gradual tapering of the strobila towards the anterior end (Figs. 12–16). The segments of *P. gadi-pollachii* vary in shape but the majority are 0·2–0·3 mm. in length and 2·0–3·0 mm. in breadth. The last 10–30, however, become gradually longer and narrower, the average dimensions of the last segment being 1·2 × 1·0 mm. (Fig. 4). The size and shape of the strobila of *A. gadi* appears to depend on the degree of infestation and possibly on host-distribution. Only one specimen of *A. gadi*, measuring 430 mm. in length, was found in the intestine of one cod examined. Anteriorly this worm was circular in transverse section, being 0·5 mm. in diameter, while posteriorly it was oval, being 3 mm. in breadth and 1·2 mm. in depth (Fig. 16). In another cod two worms recovered from the intestine and intestinal caeca were identical in size and were approximately 300 mm. in length in the living condition but contracted to 200 mm. when fixed in 4% formaldehyde. Anteriorly they were circular in transverse section, 1 mm. in diameter, and posteriorly they were oval, being 3 mm. in breadth and 2·5 mm. in depth. Six specimens from a third cod were from 100 to 130 mm. in length and circular in transverse section throughout, their entire lengths being 0·7 mm. in diameter anteriorly and 2 mm. posteriorly. The four entire specimens of *A. gadi* found in the haddock were small, the largest being 100 mm. in length. Cooper (1918) and Guiart (1935) have already indicated that specimens from the haddock are usually smaller than those from the cod. Guiart found twelve specimens of *A. gadi* in the intestine and caeca of the haddock, all less than 95 mm. long, and states that the strobila is very 'stout and almost cylindrical'. The present specimens from the haddock were also circular in transverse section throughout their lengths, and although a tapering towards the anterior end was present it was not so distinct as in specimens from the cod. An interesting feature of these four was the similarity in shape of the posterior segments to those of *Parabothrium gadi-pollachii* (Figs. 8–11). Variations of the strobila similar to those described above for *Abothrium*



Figs. 1–16. For legends see opposite page.

gadi have been reported by Markowski (1955) for some members of the Tetrabothriidae.

Longitudinal grooves or corrugations of the body wall of the strobila are characteristic features of *P. gadi-pollachii* and *A. gadi*. Seven-to-nine narrow superficial longitudinal grooves running the whole length of the strobila are present in the former species (Figs. 23, 25, 28). The grooves are V-shaped in transverse section and measure 0·008–0·23 mm. in depth and 0·08 mm. across the opening. In *A. gadi* the body wall is often corrugated but continuous longitudinal grooves are not present. Segmentation of the strobila is indistinct in both species because secondary transverse striations are strongly developed. Longitudinal grooves and corrugations have been described in other groups of cestodes, in particular in the Tetrabothriidae Braun, 1900. According to Rees (1956) the strobila of *Tetrabothrius affinis* (Lonnberg) is 'irregularly and deeply corrugated'. As far as the writer is aware the significance of these corrugations has not been discussed by previous helminthologists, and the following speculations therefore are made. It is generally accepted that a flattening of the strobila in cestodes may be an adaptation to increase the surface area in connexion with nutrition. The corrugations may be an additional step towards increasing the surface area. The grooves, as well as playing a part in the absorption of food, may possibly function as organs of attachment as, in the present instance, the two species of cestodes were sometimes found weakly adhering along their lengths to the mucosa. This is also interesting from the point of view of nutrition in the Cestoda. According to Read (1956) *Hymenolepis diminuta* is capable of deriving benefit from glucose at very low concentrations and he is of the opinion that this suggests, but does not prove, that a process of 'active absorption' may be involved as is thought to occur in the absorption of glucose by the intestinal mucosa. *P. gadi-pollachii* and *A. gadi* would benefit by using longitudinal grooves or corrugations to adhere to the intestinal villi and to increase the absorptive area. Chandler, Read & Nicholas (1950), working on *Hymenolepis diminuta*, have shown that this species may derive oxygen, nitrogenous material and vitamins directly from the host tissues, these substances being secreted into the intestinal lumen. They have also demonstrated that a high infestation may result in the worms becoming more and more in contact with one another and less and less with the mucosa and that this results eventually in poor growth of the parasite. The same factors would

Figs. 1–7. *P. gadi-pollachii*

Fig. 1. Entire strobila.
Fig. 2. Scolex deformatus, first stage in degeneration.
Fig. 3. Mid-strobila region.
Fig. 4. Posterior tapering region of strobila.
Fig. 5. Scolex deformatus, second stage in degeneration.
Fig. 6. Scolex deformatus, third stage in degeneration.
Fig. 7. Scolex deformatus, mode of attachment within pyloric caecum.

Figs. 8–16. *A. gadi*

Figs. 8–11. Specimen from the haddock showing various regions of the strobila.
Figs. 12–16. Specimen from the cod showing various regions of the strobila.

probably account for a poor growth in heavy infestations of *P. gadi-pollachii* and *A. gadi*.

(*b*) *Scolex deformatus.* Sixteen specimens of *P. gadi-pollachii* were found, and in order to investigate the anterior extremity of the strobila six of these were carefully dissected from the caecum at the time of collecting while the others were fixed in the caeca. All the specimens have been examined, some in the form of whole mounts and some were cut serially. Of those fixed *in situ* some were sectioned together with the caecum or intestine in which they were embedded.

The usual type of scolex of the Pseudophyllidea, namely with two bothria, was not present. The anterior end had degenerated to form a scolex deformatus, the specimens indicating clearly that up to 30 mm. of this end of a strobila measuring 300 mm. may disappear (Figs. 2, 5, 6). The portion of the worm within the caecum was variable in size, shape and colour, depending probably on the state of contraction and stage of degeneration of the anterior end during the formation of a scolex deformatus.

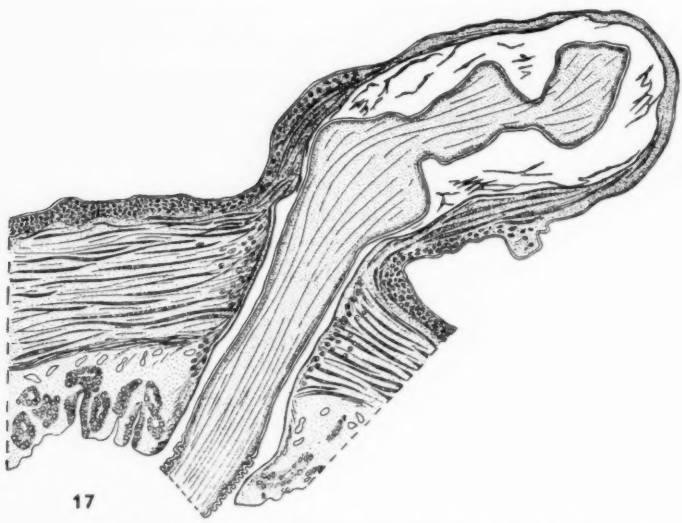


Fig. 17. *P. gadi-pollachii*, section through the scolex deformatus embedded in the wall of the intestine.

The scolex deformatus measures approximately 13–15 mm. in length, 3·0–3·5 mm. in breadth and possesses a permanent bulbous swelling 3·5–4·0 mm. in diameter near its base. This swelling may play an important part in wedging the scolex deformatus inside the caecum, an unusual but very interesting mode of attachment for a cestode (Fig. 7). The same swelling occurred in the specimen embedded in the intestinal wall (Fig. 17). A mode of attachment not unlike that described here is known for some other species of the Cestoda, for example *Priapocephalus grandis* Nybelin, 1922, the anterior extremity of which is embedded in the

host's intestine, and for *Strobilocephalus triangularis* (Diesing, 1850), redescribed by Baer (1955).

The scolex deformatus varies in colour from dark brown apically to a creamy white basally (Fig. 2). A similar colour variation is seen on the external wall of a caecum containing specimens of *P. gadi-pollachii* (Fig. 7). It is an easy matter therefore to determine whether caeca contain specimens of the cestode. The

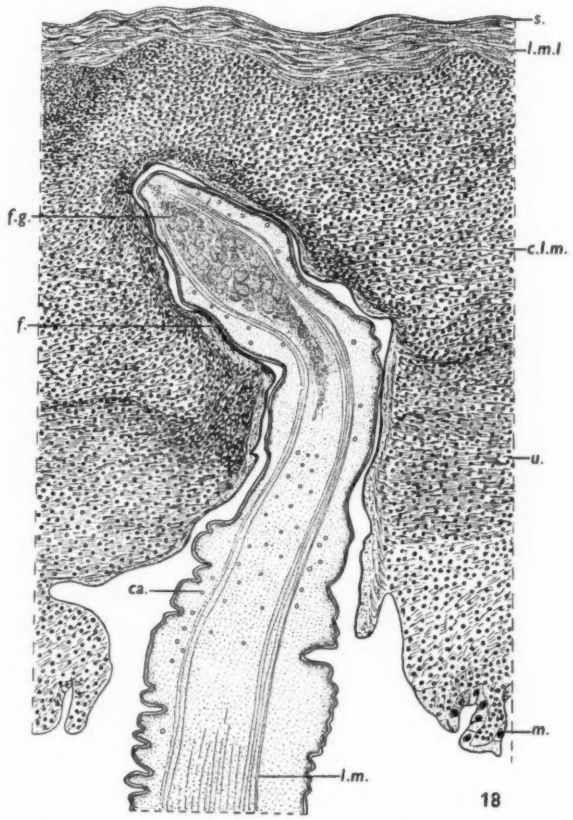
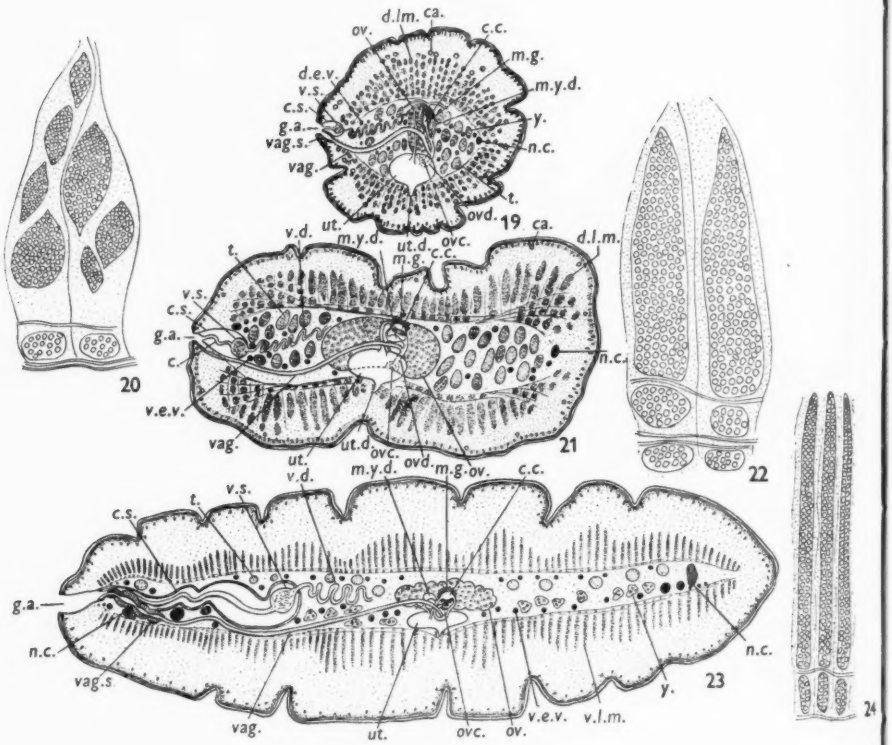


Fig. 18. *A. gadi* from the haddock, saggital section through the anterior end embedded in the wall of the intestine.

variation in colour of the host's caecum is probably due to some chemical change in the host tissues resulting from a host/parasite reaction. In an advanced state of parasitism the wall of the caecum disappears completely, the anterior end of the worm protruding into the body cavity. In some cases it was found that the scolex deformatus was firmly attached to the lining of the caecum, but it could not be determined whether or not this adhesion was the result of a glandular secretion from the parasite as no gland cells could be detected in this region. In *A. gadi*, unlike *P. gadi-pollachii*, a well-developed scolex deformatus is not present.

There is, however, an indication that a degeneration of the anterior end does take place. Many specimens from the cod were firmly attached within the caeca in the same manner described for *P. gadi-pollachii*. It has been stated above that the scolex deformatus, which is not homologous with the scolex of the Pseudophyllidea, is a modification of the anterior end of the strobila. As was anticipated, therefore, there is no extreme modification of the muscular, nervous and excretory systems within it. The deep longitudinal muscle bundles on entering the



Figs. 19–22. *A. gadi*

Fig. 19. Specimen from the haddock, transverse section in mid-strobila region showing deep longitudinal muscles, nervous and reproductive systems.

Fig. 20. Specimen from the haddock, showing detail of deep longitudinal muscle bundles.

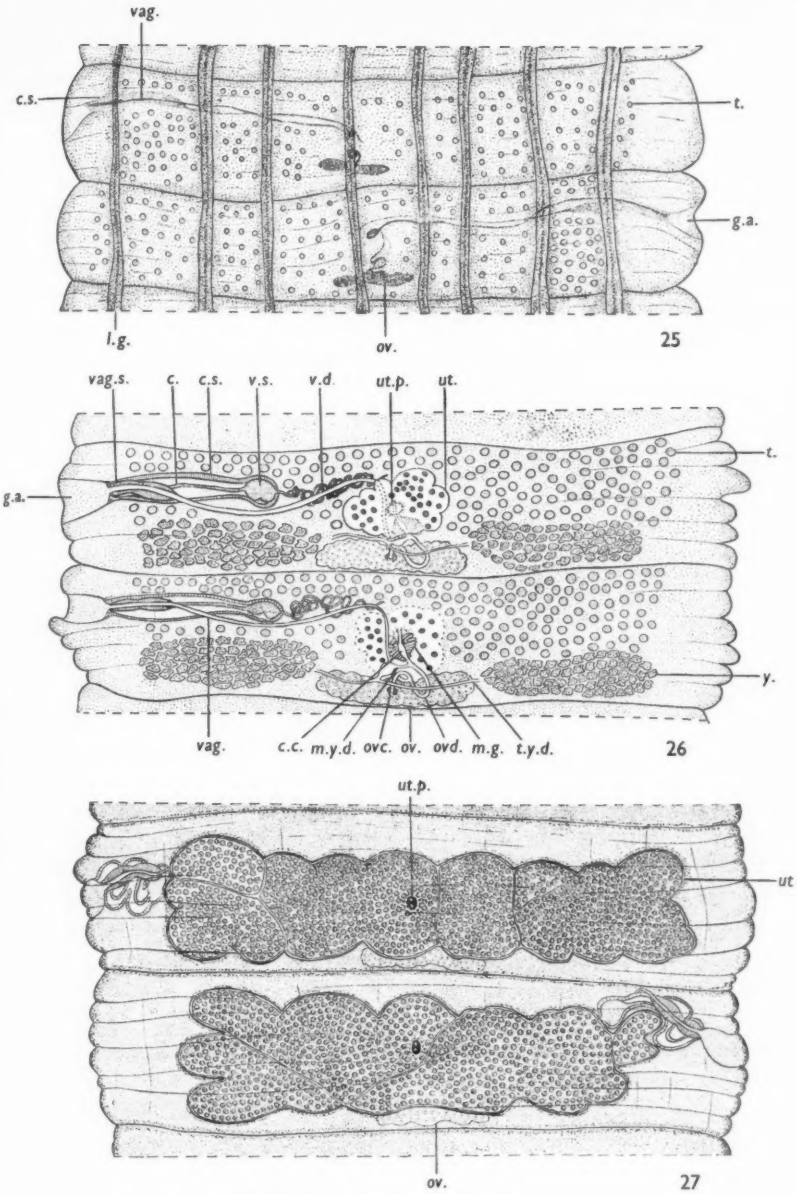
Fig. 21. Specimen from the cod, transverse section in mid-strobila region showing deep longitudinal muscles, nervous and reproductive systems.

Fig. 22. Specimen from the cod, showing detail of deep longitudinal muscle bundles.

Figs. 23, 24. *P. gadi-pollachii*

Fig. 23. Transverse section in mid-strobila region showing deep longitudinal muscles, nervous and reproductive systems.

Fig. 24. Detail of deep longitudinal muscle bundles.



Figs. 25–27. *P. gadi-pollachii*

Fig. 25. Anterior region in ventral view showing corrugations of body wall and rudiments of the genitalia.

Fig. 26. Mid-strobila region showing male and female genitalia.

Fig. 27. Gravid segments of posterior region of the strobila. The corrugations of the body wall are not shown in Figs. 26 and 27.

pseudo-scolex disperse into individual fibres which, as Woodland (1927) has stated, 'penetrate the whole parenchymal mass to the apex' (Fig. 17). The excretory vessels and lateral nerves are also unmodified from the condition in the strobila.

When either of these two species is embedded in the intestinal wall the latter becomes damaged and discoloured. The following observations have been made on the damaged intestine (Fig. 18). Five layers could be made out in a section of the intestine, but one of these could not be identified. The serosa and longitudinal muscles, the two outermost layers of the wall, appear to be unaffected by the parasite. The next layer is a layer of circular muscle which has undergone extreme fibrosis, a fibrous layer being exceptionally well marked in the immediate neighbourhood of the parasite. The circular muscle layer is followed by the layer which

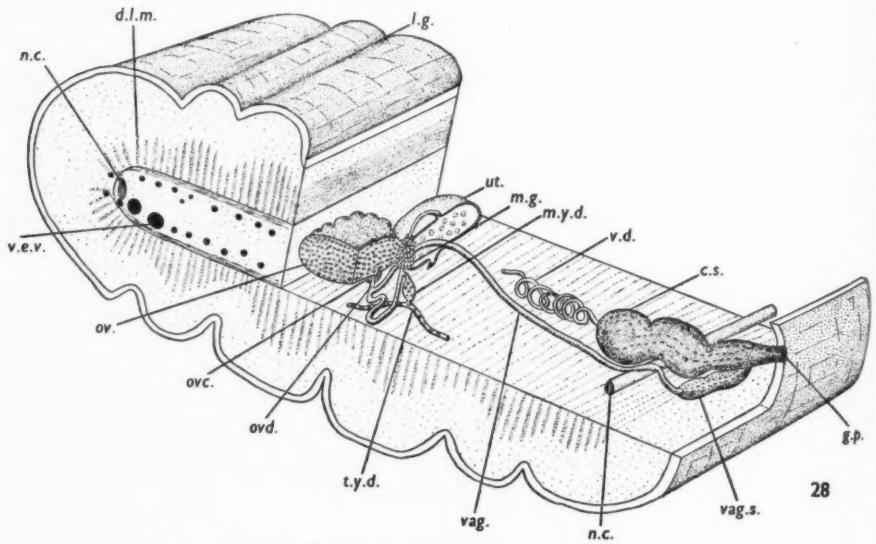


Fig. 28. *P. gadi-pollachii*, diagram to show the general arrangement of the muscular, excretory, nervous and genital systems.

has not been identified because of the extreme fibrosis. According to Al-Hussaini (1949) there is much variation in the detailed histology of the teleost intestine. The unidentified layer may have been unstriated circular muscle fibres. It may have been longitudinal muscles next to the submucosa as, according to Li (1937), such a layer is present in some teleosts, e.g. the common carp. The mucosa and submucosa could be seen in this section but the latter had undergone much fibrosis.

Musculature. The musculature of the strobila will not be described in detail because the general arrangement is similar to that described for other members of the Pseudophyllidea, and the writer also is in agreement with Woodland's (1927) observations on this system in *P. gadi-pollachii*. At first sight the arrange-

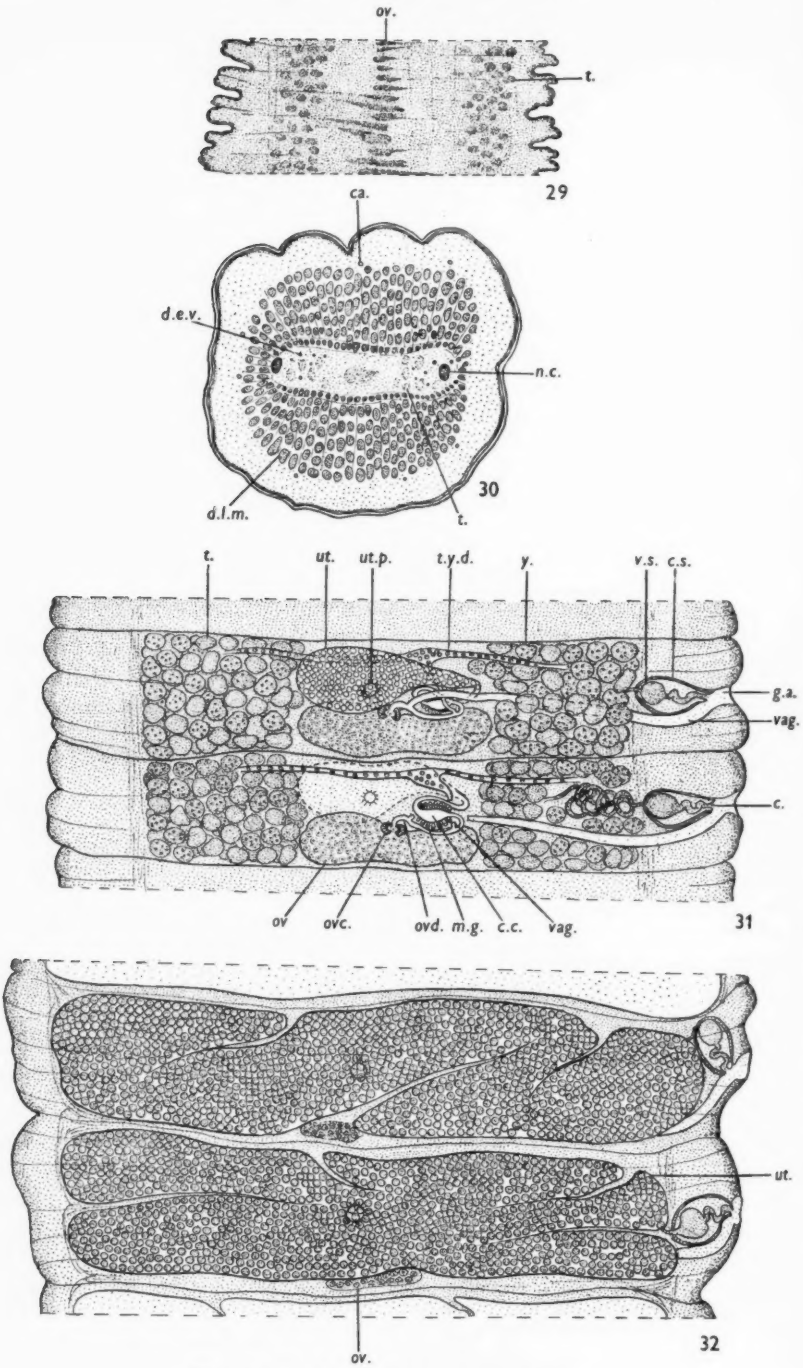
ment and size of the deep longitudinal muscles in *A. gadi* appear to be of great taxonomic value, but as has already been shown by Williams (1959) in the Phyllobothriidae Braun, 1900, variations may occur in different regions of the same individual as can be seen particularly in a comparison of Figs. 21 and 30. The number of fibres constituting each bundle varies according to the size of the bundle. This is shown in Figs. 19–24. While considering the musculature it might be noted that the spherical calcareous corpuscles in the cortex of *A. gadi* is a conspicuous characteristic feature of all the specimens examined (Figs. 19, 21). They have not been seen in *P. gadi-pollachii*, but according to Woodland (1927) calcareous corpuscles are present in immature individuals of this species.

Excretory system. The excretory system in the two species appears to be confined to the medullary region of the strobila (Figs. 19, 21, 23). The number of longitudinal excretory vessels seen in transverse sections throughout the length of an individual varies from 23 to 50 in *P. gadi-pollachii* and from 10 to 25 in *A. gadi*. The differences suggest that the vessels are branched, but the writer has not been able to detect any branching. The diameter of the vessels also varies from 0·045 to 0·075 mm. in *P. gadi-pollachii* and from 0·006 to 0·015 mm. in *A. gadi*. There appears to be some pattern in their distribution in the former species as in the majority of sections examined there are from two to four large ventro-lateral vessels on each side. Of the remainder the ventral are always slightly larger than the dorsal vessels (Fig. 28).

Nervous system. All that has been seen of the nervous system in both species are two lateral, longitudinal nerve cords, one on each side of the body along the margin of the medulla (Figs. 19, 21, 23). The nerves run from the anterior to the posterior extremity of the strobila and may be dorsal or ventral to the cirrus sac. In *P. gadi-pollachii* the nerve cords measure 0·060 × 0·030 mm. in transverse section, while in *A. gadi* from the cod and haddock they measure 0·090 × 0·045 and 0·075 × 0·045 mm. respectively.

Genitalia. The genital atrium in both species is lateral, irregularly alternate and situated mid-way in the length and depth of each segment (Figs. 28, 36). In *P. gadi-pollachii* the depth of the atrium is always at least three times that of *A. gadi* (Figs. 19, 21, 23). In the latter species from the cod the diameter of the opening is 0·08 mm. and the depth 0·08–0·10 mm. In *A. gadi* from the haddock comparative measurements are 0·06 and 0·05–0·06 mm.

(*a*) *Male genitalia.* In *P. gadi-pollachii* there are 120–140 testes, which are mainly dorsal, and in two fields (Fig. 25). Rudiments of the testes, 0·025 mm. in diameter, appear in the region immediately behind the pseudoscolex. In the middle region of the strobila the testes attain a maximum diameter of approximately 0·045 mm. (Fig. 26). Vasa efferentia have not been observed. The much-coiled vas deferens is slightly anterior, dorsal and to the left or right of the middle line depending on the position of the genital atrium. It is always packed with sperms and probably functions as a vesicula seminalis. Rudiments of the cirrus can be seen in the segments immediately behind the pseudoscolex (Fig. 25), and it seems to be fully developed in those situated in the middle region of the strobila (Fig. 26), where it is roughly club-shaped and attains a size of 0·725 mm. in length



Figs. 29–32. For legends see opposite page.

and 0·15 mm. in maximum diameter. The internal vesicula seminalis is roughly bean-shaped, measuring 0·15 × 0·06 mm., and is continuous with the cirrus, which is straight and unarmed, measuring 0·77 mm. in length and 0·05 mm. in diameter. The cirrus opens into the dorsal side of the genital atrium.

In *A. gadi* the arrangement of the male genitalia is similar to that of *P. gadi-pollachii* but differences occur in the distribution, number, size and shape of the testes, cirrus sac and vesicula seminalis (Figs. 26, 31, 34). There are also differences in distribution, size and number of the testes in specimens of *A. gadi* from the cod and haddock. In those from the cod there are 100–120 spherical to oval testes measuring approximately 0·07–0·11 mm. in diameter (Fig. 31). They are mainly dorsal and situated in two lateral fields. In the same species from the haddock there are approximately fifty testes which are oval in shape, the long axis of the oval being vertical (Figs. 19, 36). They are relatively much larger than in the species from the cod, measuring approximately 0·125 × 0·075 mm., and are not situated in two lateral fields. In *A. gadi* from the cod (Fig. 31) the cirrus pouch is 0·016–0·24 mm. long and 0·11–0·14 mm. in diameter, the vesicula seminalis being spherical, measuring 0·09 mm. in diameter, and the unarmed cirrus is 0·14–0·16 mm. long and 0·03–0·04 mm. in diameter. In the same species from the haddock (Figs. 34, 36) the cirrus pouch is not more than 0·17 mm. long and 0·06 mm. in diameter, the unarmed cirrus being 0·09–0·12 mm. long and 0·02–0·03 mm. in diameter and the vesicula seminalis 0·05 mm. in diameter.

(*b*) *Female genitalia.* In *P. gadi-pollachii* the ovary which is in the medulla is posterior, dorsal and in the middle line in each segment (Fig. 26). It varies in size from 0·075 mm. in length, 0·225 mm. in breadth and 0·075 mm. in depth in the immature segments (Fig. 25) to 0·95 × 0·85 × 0·12 mm. in the mature region (Fig. 26). It is irregularly lobed, the oviduct arising as an ovicapt centrally on its ventral side (Fig. 28). The ovicapt is very muscular, measuring 0·060 mm. long and 0·045 mm. in maximum diameter. The oviduct receives the vagina near the ovicapt and continues ventrally before turning anteriorly and dorsally. It receives the median yolk-duct near the level of the dorsal side of the ovary and then becomes slightly swollen to form the central chamber, which is surrounded by Mehlis's glands. The oviduct emerges from the gland dorsally and continues as the uterine duct anteriorly for a short distance before turning ventrally to open into the dorsal side of the uterus. The latter is at first a small, median, sac-like structure but becomes much enlarged and folded towards the posterior end of the strobila. A wide, blindly ending diverticulum extends from the centre of its ventral side (Fig. 37), the size of this depending on the stage of development of the uterus. In a fully gravid segment the diverticulum may extend as far as the cuticle of the

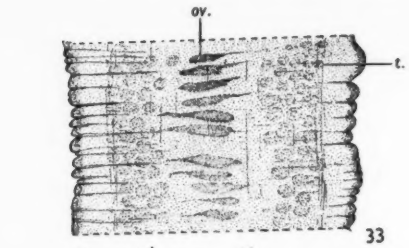
Figs. 29–32. *A. gadi* from the cod

Fig. 29. Anterior region of strobila showing rudiments of male and female genitalia.

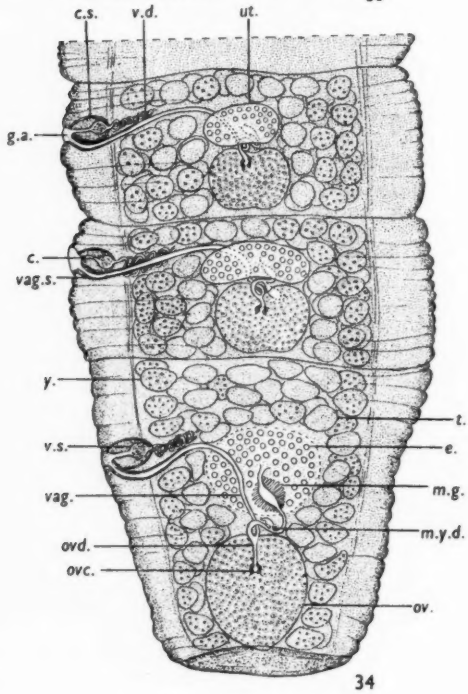
Fig. 30. Transverse section showing the arrangement of the deep longitudinal muscles in the anterior region of the strobila.

Fig. 31. Mid-strobila region showing male and female genitalia in ventral view.

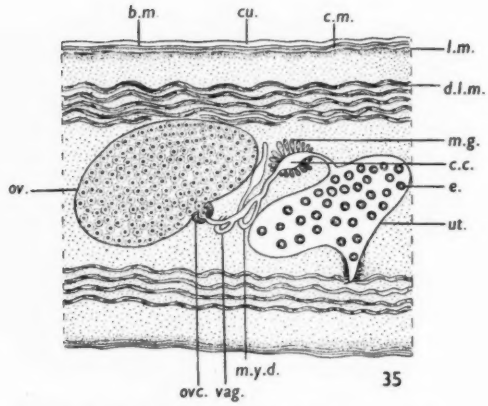
Fig. 32. Posterior gravid region of the strobila in ventral view.



33



34



35

Figs. 33–35. For legends see opposite page.

body wall, but does not communicate with the exterior in any of the present specimens. Occasionally there is a corresponding indentation of the cuticle directed towards the diverticulum, but the two are never continuous even in fully gravid proglottides. According to Wardle & McLeod (1952) a uterine pore is present in *P. gadi-pollachii*, appearing when eggs begin to accumulate in the uterus. Serial sections of the uterus, however, show that a true uterine pore may not always be present and that the uterus eventually opens to the exterior by many pores due to a rupture of the body wall in several places. This condition is interesting when compared with some members of the Tetrabothriidae. According to

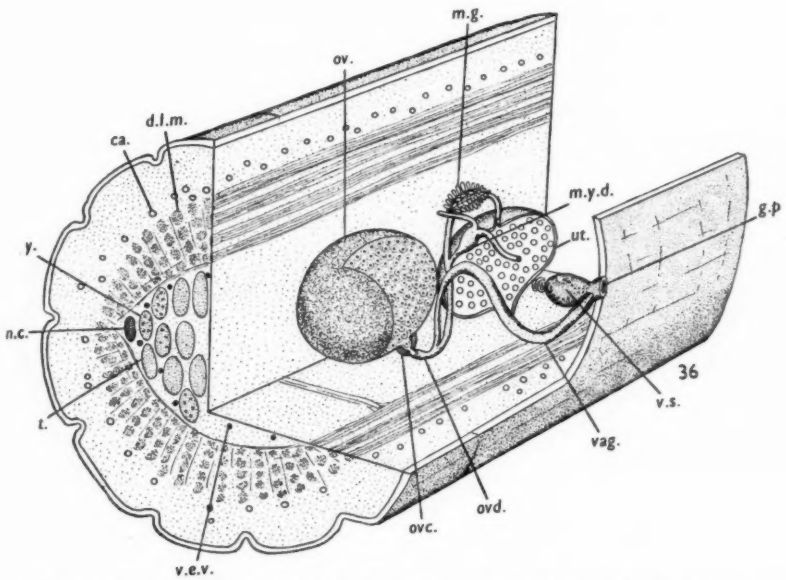


Fig. 36. *A. gadi* from the haddock, diagram to show the general arrangement of the muscular, excretory, nervous and genital systems.

Markowski (1956) there is no true uterine pore in some members of this family. the eggs being discharged through ruptures in the body wall which appear on the dorsal surface. In *P. gadi-pollachii* the gravid uterus, which is packed with eggs, occupies the medullary region and forces aside all other structures in the medulla (Fig. 27). The dorso-ventral muscles, however, retain their connexions in many places and the uterus pushes its way between them, and in whole mounts and sections therefore it appears as a slightly reticulate structure.

Figs. 33–35. *A. gadi* from the haddock

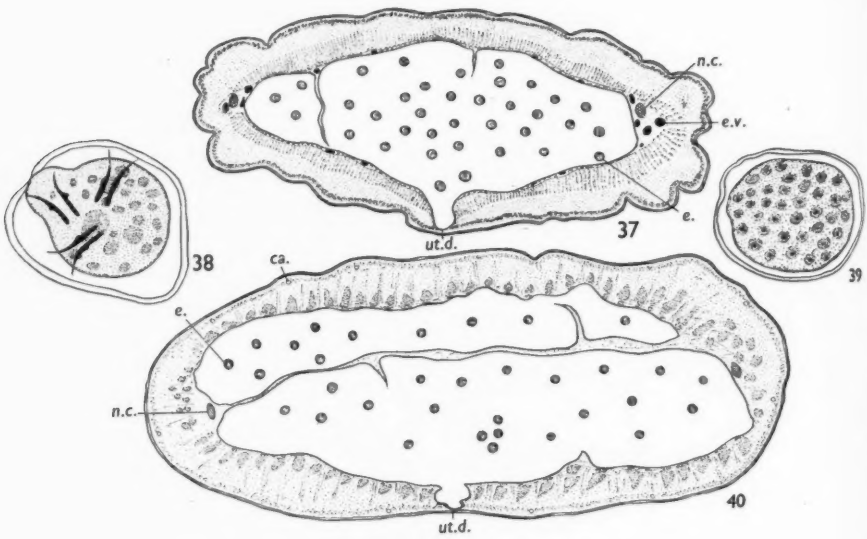
Fig. 33. Anterior region of strobila showing rudiments of male and female genitalia in ventral view.

Fig. 34. Segments from the posterior region showing male and female genitalia in ventral view.

Fig. 35. Median sagittal section of a segment showing male and female genitalia.

The eggs are spherical, measuring 0·035–0·037 mm. in diameter, and contain embryos which are variable in size (Fig. 38). There are three pairs of embryonic hooks measuring 0·006 mm. in length. A characteristic feature is the apparent absence of ciliated membrane around the embryo. The eggs are also interesting when compared with some Tetrabothriidae because they appear to be almost identical with the eggs described by Markowski (1956) for many members of the order.

The yolk follicles are in two latero-ventral groups, one to the right and the other to the left of the ovary (Fig. 26). There are approximately forty to fifty follicles in each group, which are sometimes so closely packed that the glands assume the appearance of compact vitellaria. Each follicle is irregular in shape and measures 0·050 × 0·045 mm. The vagina, which is 0·03 mm. in diameter, runs more or less



Figs. 37, 38. *P. gadi-pollachii*

Fig. 37. Transverse section of posterior region showing uterus, false uterine pore and eggs.

Fig. 38. Detail of egg.

Figs. 39, 40. *A gadi* from the cod

Fig. 39. Detail of egg.

Fig. 40. Transverse section of posterior region showing uterus, false uterine pore and eggs.

parallel and ventral to the coiled vas deferens and cirrus sac and opens into the genital atrium slightly in front of and ventral to the opening of the cirrus. The vagina is lined with a ciliated epithelium and is provided with a very powerful terminal sphincter muscle 0·023 mm. in length and 0·08 mm. in diameter. The wall of the sphincter is 0·03 mm. in thickness (Fig. 23).

The female genitalia of *A. gadi* resemble those of *P. gadi-pollachii* in general arrangement but a few differences are considered below (Figs. 26, 31, 34). In specimens from the cod the ovary (Fig. 31) is transversely elongated, roughly kidney-shaped and usually with a smooth surface. In those segments in the middle region of the strobila it attains a maximum size of 0·3 mm. in length, 0·80 mm. in breadth and 0·30 mm. in depth. The oval vitelline follicles are scattered in the medulla amongst the testes and on each side of the middle line. The ovary varies in shape in *A. gadi* from the haddock (Figs. 35, 36), being spherical usually, but in the last segment it may be roughly egg-shaped with the long axis longitudinal. The ovicapt is mid-ventral and slightly anterior in position. Other features of the female genitalia, except for the vagina, resemble those of *A. gadi* from the cod. The terminal part of the vagina, however, appears to be supplied with a weakly developed sphincter, its wall being 0·015 mm. in thickness (Figs. 19, 36). The eggs and embryos (Figs. 39, 40) of *A. gadi* have been described by Yamaguti (1934). As the writer's interpretation of the structure of the egg appears to agree with that of Yamaguti an account of the morphology will not be repeated here.

DISCUSSION

Several interesting points have arisen as a result of the comparison of *P. gadi-pollachii* and *A. gadi*, both of which are classified, as a general rule, as members of the family Amphicotylidae. Some of these features have already been discussed in the foregoing account, but those which involved long discussions are given below.

It was very difficult to distinguish the two species, and this distinction was made even more difficult by the variations that occur in *A. gadi*. These are a wide variation in size and shape of the segments, size, shape and number of testes, size and shape of the ovary, the presence of a vaginal sphincter in *A. gadi* from the haddock and the apparent variation in the deep longitudinal muscles. In some groups of the Pseudophyllidea specific and even a generic determination is sometimes very difficult because variations similar to those described in the present instance have been found in other families of this order, for instance the Diphyllobothriidae. Stunkard (1948) was unable to assign to a genus four cestodes of this family from Pinnipedia caught in Alaska. He was of the opinion that many descriptions of cestodes from seals and sea lions are so incomplete and inadequate that it is impossible to allocate specimens to previously described species until detailed descriptions of them have been made. Markowski (1949) listed nine possible specific criteria for the genus *Diphyllobothrium*, including the shape of the segments, the number, size, shape and disposition of the testes and the shape of the ovary. He also stated that the number of testes is 'very unreliable as a systematic criterion, because in the same species and even in a single worm the maximum number may be twice as great as the minimum', and pointed out that the dimensions of the testes and the shape of the ovary vary according to their stage of development. Rausch (1956) is of the opinion that because a wide range of morphological variation is always expected in species of the genus *Diphyllobothrium* they cannot be differentiated effectively through use of adult morphological features

alone, and he has shown that the plerocercoid larva, host species, habitat and morphology of the species should be studied in detail.

In view of the above remarks the material of *A. gadi* from the cod and haddock cannot be segregated into two species in spite of the variations found in individuals from these two hosts. Stunkard (1957) has already given a most interesting discussion of the vast variation shown by a single species developing in different hosts. It follows therefore that detailed descriptions of members of the Pseudophyllidea are necessary to facilitate identification in future. Brief accounts will only result in a state of chaos as is already the case in the Diphyllobothriidae.

A scolex deformatus is a characteristic feature of the adult of *P. gadi-pollachii*. This type of scolex and its mode of attachment is not unlike that of *Priapocephalus*, a member of the Tetrabothriidae described by Nybelin (1922), Baylis (1926), Markowski (1955) and Baer (1954). The similarity, according to some helminthologists, indicates a relationship between the Tetrabothriidae and the Pseudophyllidea. Baer (1932), however, stated that the scolex of *Priapocephalus* has nothing in common with the scolex deformatus found in some members of the Pseudophyllidea. In *Parabothrium* the scolex deformatus is a modification of the strobila, sometimes to the extent of 30 mm. behind the anterior end of the larva, and it is not therefore homologous with a scolex possessing two bothria, which is characteristic of the Pseudophyllidea. Whether or not such a degeneration occurs in *Priapocephalus* is not known. According to Baer (1954) no young specimens of the latter have been found, and it is not known therefore whether the scolex of the adult worm is derived from the classic type of scolex found in the Tetrabothriidae. He states that if young forms are found which possess two bothria the genus *Priapocephalus* may have to be included in the Pseudophyllidea.

According to Olsson (1866) there is a slight indication that two bothria may be present in young stages of *A. gadi*. In all other previous descriptions of *P. gadi-pollachii* and *A. gadi* there has been very little evidence to show, even when young stages have been described, that two bothria are present in these two species. Johnstone (1907), Scott (1909) and Cooper (1918) have expressed doubt regarding the presence of two bothria in *A. gadi*. It is true that in some of the present specimens of *A. gadi* and in those examined by Cooper (1918) a small slit-like invagination occurs at the apex of the anterior end, but it should not be concluded that it represents a stage in the degeneration of a bothrium. A similar invagination has been figured by Fuhrmann (1931) in the genus *Priapocephalus*. Stages in the degeneration of two bothria have been found in other members of the Amphicotylidae, for instance in the genus *Eubothrium*, but for this reason it cannot be assumed that two bothria have degenerated in other members of the family. One of the specimens of *A. gadi* examined shows very slight evidence that two bothria are absent even in the young stage of this species. It was found embedded in the intestinal wall of the haddock and appears to possess a mass of cells in the centre of the anterior extremity (Fig. 18). Whether these are gland cells, homologous and analogous with the frontal glands of plerocercoid larvae is difficult to ascertain at present. In that portion of the strobila embedded in the intestine there is no indication of the presence of two bothria, and since there is no sign of degeneration

of the anterior end it is possible that two bothria are absent in *A. gadi*. If this is confirmed it may have an important bearing on the relationships of the Amphicotylidae with other cestodes.

SUMMARY

An account is given of the occurrence of *Parabothrium gadi-pollachii* and *Abothrium gadi* in some members of the Gadidae caught in the north-eastern Atlantic.

The external features, musculature, excretory, nervous and genital systems of the two species are described in detail.

The adults of both species are characterized by the absence of a typical pseudophyllidean scolex and possess interesting modes of attachment to the intestine and caeca of the host. The variation found in individuals of *A. gadi* from the cod and haddock is discussed.

The writer is indebted to Dr Gwendolen Rees for her invaluable guidance in the preparation of the above work, to Professor T. A. Stephenson, F.R.S., for his interest and encouragement, and to the Department of Scientific and Industrial Research who made the investigation possible by giving generous financial aid.

REFERENCES

Baer, J. G. (1932). Contribution à l'étude des Cestodes de Cétacés. *Rev. suisse Zool.* **39**, 195–228.

Baer, J. G. (1954). Revision taxonomique et étude biologique des cestodes de la famille des Tetrabothriidae, parasites d'oiseaux de haute mer et de Mammifères marins. *Mém. Univ. Neuchâtel*, **1**, 121 pp.

Baer, J. G. (1955). Cestodes d'un dauphin de l'océan Pacifique. *Bull. Soc. neuchâtel Sci. nat.* **78**, 33–36.

Baylis, H. A. (1926). Some tetrabothriid cestodes from whales of the genus *Balaenoptera*. *J. Linn. Soc.* (*Zool.*), **36**, 161–72.

Brown, W. W. & Cheng, C. (1946). Investigations into the food of the cod (*Gadus callarias* L.) off Bear Island and of the cod and haddock (*G. aeglifinus* L.) off Iceland and the Murman coast. *Hull Bull. mar. Ecol.* **3**, 35–71.

Chandler, A. C., Read, C. P. & Nicholas, H. O. (1950). Observations on certain phases of nutrition and host parasite relations of *Hymenolepis diminuta* in white rats. *J. Parasit.* **36**, 523–35.

Cooper, A. R. (1918). North American pseudophyllidean cestodes from fishes. *Illinois biol. Monogr.* **4**, 154–70, 186–200.

Dollfus, R. Ph. (1953). Parasites animaux de la Morue Atlanto-Artique. *Encycl. biol.* **43**, 1–482.

Fuhrmann, O. (1931). Dritte Klasse des Cladus Plathelminthes. Cestoidea. *Handb. Zool. Kükenthal u. Krumbach.* **2**, 141–416.

Guiart, J. (1935). Cestodes parasites provenant des campagnes scientifiques de S.A.S. le Prince Albert der de Monaco (1886–1913). *Résult. Camp. sci. Monaco*, **91**, 1–100.

Heyneman, D. (1959). Cuticular peeling: a dissection technique for preparation of cestode whole mounts. *J. Parasit.* **45**, 573–4.

Al-Hussaini, A. H. (1949). On the functional morphology of the alimentary tract of some fish in relation to differences in their feeding habits: anatomy and histology. *Quart. J. Micr. Sci.* **90**, 1–39.

Jenkins, T. (1950). *The Fishes of the British Isles*, pp. 1–408. New York: Warne and Co. Ltd.

Johnstone, J. (1907). Internal parasites and diseased conditions of fishes. Cestoda, Trematoda and Protozoa. *Proc. Lpool Biol. Soc.* **21**, 270–303.

Li, P. L. (1937). Neue Beobachtungen über die Strukter des Zirkulärmuskels im Dünndarm bei Wirbeltieren. *Z. Anat. Entw.Gesch. Berl.* **107**, 212.

Markowski, S. (1949). On the species of *Diphyllobothrium* occurring in birds and their relation to man and other hosts. *J. Helminth.* **23**, 107–26.

MARKOWSKI, S. (1955). Cestodes of whales and dolphins from the Discovery collections. *Discovery Rep.* **27**, 377–95.

NYBELIN, O. (1922). Anatomisch systematischen Studien ueber Pseudophyllidien. *Göteborgs. Vetenskapsakad. Handl.* **26**, 169–211.

OLSSON, P. (1866). Entozoa iakttagna hos Skandinaviska hafsfiskar. *Acta Univ. Lund. math. natur-vetensk.* **4**, 1–59.

RAUSCH, R. (1954). Studies on the helminth fauna of Alaska. XVI. Taxonomy, morphological variation and ecology of *Diphyllobothrium ursi* n.sp. provis. On Kodiak Island. *J. Parasitol.* **40**, 540–63.

RAUSCH, R. (1955). Unusual pathogenicity of *Diphyllobothrium* sp. in a black bear. *Proc. Helminth. Soc. Wash.* **22**, 95–7.

RAUSCH, R. (1956). Studies on the helminth fauna of Alaska. XXXVIII. The description and occurrence of *Diphyllobothrium dalliae* n.sp. (Cestoda). *Trans. Amer. Micr. Soc.* **75**, 180–7.

READ, C. P. (1956). Carbohydrate metabolism of *Hymenolepis diminuta*. *Exp. Parasitol.* **5**, 325–44.

REES, G. (1956). The scolex of *Tetrabothrius affinis* (Lonnberg), a cestode from *Balaenoptera musculus* L., the blue whale. *Parasitology*, **46**, 426–42.

SCOTT, T. (1909). Some notes on fish parasites. *Ann. Rep. Fish. Bd Scot.* **26**, 73–92.

SMYTH, J. D. & JOHRI, L. N. (1956). A histochemical approach to the study of helminth morphology. *Parasitology*, **46**, 107–16.

STUNKARD, H. W. (1948). Pseudophyllidean cestodes from Alaskan Pinnipeds. *J. Parasitol.* **34**, 211–28.

STUNKARD, H. W. (1957). Intraspecific variation in parasitic flatworms. *Systematic Zoology*, **6**, 7–18.

WARDLE, R. Z. & MCLEOD, J. A. (1952). *The Zoology of Tapeworms*. University of Minnesota Press.

WOODLAND, W. N. F. (1927). On *Dinobothrium septaria* van Beneden 1889, and *Parabothrium bulbiferum* Nybelin 1922. *J. Parasitol.* **13**, 231–48.

WILLIAMS, H. H. (1959). The anatomy of *Phyllobothrium sinuosiceps* sp.nov. (Cestoda: Tetraphyllidea) from *Hexanchus griseus* (Gmelin) the six-gilled shark. *Parasitology*, **49**, 54–69.

YAMAGUTI, S. (1934). Studies on the helminth fauna of Japan, Part 4, Cestodes of fishes. *Japan J. Zool.* **6**, 1–112.

KEY TO LETTERING OF TEXT-FIGURES

b.m.	basement membrane	*m.*	mucosa
c.	cirrus	*m.g.*	Mehlis's gland
ca.	calcareous corpuscle	*m.y.d.*	median yolk duct
cae.	caecum	*n.c.*	nerve cord
c.c.	central chamber	*ov.*	ovary
c.l.m.	circular and longitudinal muscle layer	*ovc.*	ovicapt
		ovd.	oviduct
c.m.	circular muscle	*s.*	serosa
c.s.	cirrus sac	*s.d.*	scolex deformatus
cu.	cuticle	*t.*	testis
d.l.m.	deep longitudinal muscles	*t.y.d.*	transverse yolk duct
d.e.v.	dorsal excretory vessel	*u.*	unidentified layer of tissue
e.	egg	*ut.*	uterus
e.v.	excretory vessel	*ut.d.*	uterine duct
f.	fibrous layer	*ut.p.*	false uterine pore
f.g.	'frontal gland'	*v.d.*	vas deferens
g.a.	genital atrium	*v.e.v.*	ventral excretory vessel
g.p.	genital pore	*v.l.m.*	ventral longitudinal muscles
i.	intestine	*v.s.*	vesicula seminalis
l.g.	longitudinal groove in body wall	*vag.*	vagina
l.m.	longitudinal muscles	*vag.s.*	vaginal sphincter
l.m.l.	longitudinal muscle layer	*y.*	yolk gland.

Parasitology (1960), **50**, 323–327
With 5 text-figures
Printed in Great Britain

Note on host-parasite relationships observed in fluke infections of wild aquatic birds

By B. P. PANDE, S. S. AHLUWALIA and J. S. SRIVASTAVA
Department of Parasitology, U.P. College of Veterinary Science and Animal Husbandry, Mathura

(*Received* 5 *August* 1959)

The last twenty-five years have witnessed an increasing output of significant work in this country on the trematode parasites of a number of wild birds, including aquatic species. These studies, which are mostly faunistic, and can be said to have commenced in 1926, when Bhalerao (1926) described from Burma (then a province of India) the flukes of the house crow, deal primarily with a large number of representative species of nearly twenty families of Digenea. Of these families, species assignable to the Clinostomidae Lühe, 1901, the Cyathocotylidae Poche, 1926, the Cyclocoelidae Kossack, 1911, the Diplostomidae Poirier, 1886, the Echinostomatidae Poche, 1926, the Notocotylidae Lühe, 1909, the Opisthorchiidae Braun, 1901, and the Strigeidae Railliet, 1919, were encountered in a survey conducted to assess the nature of helminthic infections in wild aquatic birds of the Mathura area.

The studies so far conducted in this country report the occurrence of forms, some of which are already known from other countries, or they describe other forms designated as new species. Some of the papers published during the last twenty-five years also contain remarks and discussions, based on adult characters and on the affinities and classification of the forms described. Notable among these papers are those of Mehra and his pupils from Allahabad, and those of Thapar and his associates and students from Lucknow and Hyderabad.

In spite of this large volume of published work, the knowledge of the life-cycles and pathogenic effects of the species discussed remains comparatively meagre and a great field of work lies unexplored. In the present work it was possible to ascertain, histologically, in two of the species collected, the nature of the host-parasite relationship and the present communication records, for the first time, a few details regarding an echinostomatid and a cathaemasid infection in two host species, *Xenorhynchus asiaticus*, the black-necked stork ('Loharjang, Loha Sarang'), and *Ibis laucocephalus*, the painted stork ('Janghil'). The echinostomatid species can be assigned to the genus *Echinochasmus* Dietz, 1909, and the Cathaemasid is a species of *Cathaemasia* Looss, 1899. It may be mentioned here that *X. asiaticus* and *Ardea goliath* have been recorded as the hosts of *Cathaemasia dollfusi* Travassos (1951) (syn. *C. hians* Dubois, 1950). A full paper on the complete survey will be published separately.

Cathaemasia sp.

Five immature specimens of this form were collected from the proventriculus and the oesophagus of the black-necked and the painted storks available for examination. The youngest specimen (Fig. 1), found lying free in the lumen of the proventriculus, was studied from a permanent stained preparation. It was 1·4 × 0·5 mm. in size and had a fleshy body of a reddish colour and a spinose ventral surface. Its oral sucker was 0·1 mm. in diameter and was followed by a pharynx 0·12 mm. in diameter, leading into an oesophagus, 0·24 mm. long. The intestinal bifurcation lies at one-third of the total body length and the caeca,

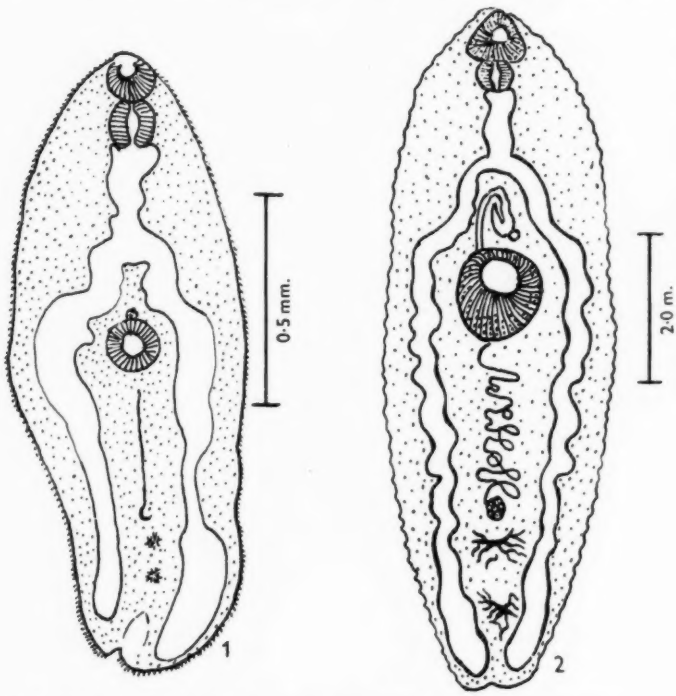


Fig. 1. An immature cathaemasid, found lying free in the proventriculus.

Fig. 2. An immature cathaemasid, extracted out of the glands of the proventriculus.

which reach nearly to the posterior end, distinctly show, between their tips, a portion of the excretory bladder. At this stage of development rudiments of genitalia may be clearly noticed in the intercaecal space, all of them lying in the median plane. The acetabulum, 0·13 mm. in diameter, lies immediately in front of the middle of the body. The two post-ovarian testes are branched and tandem and the ovary is connected anteriorly with a straight uterine tube. The genital pore lies just in front of the acetabulum.

Four other specimens, further developed than the one described above, were extracted out of the glands of the proventriculus. Two of these, together with the

surrounding tissue, were preserved for histological studies. The others were, after flattening, stained and permanently mounted. The latter are 4·5 × 1·8 mm. in size and characteristically have a more powerful acetabulum, the ratio between it and the oral sucker being 2:1. The other distinctive developmental features are the greater size of the testes, ovary and uterus and the appearance of a cirrus sac (Fig. 2). Study of the serial sections (Fig. 3) shows this stage lying inside the enlarged gland and nearly filling up its lumen, its powerful acetabulum drawing in a portion of the glandular epithelium as a plug. The affected gland shows pressure

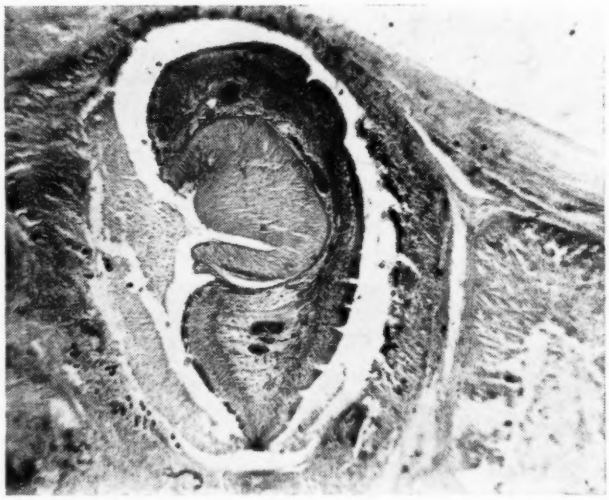


Fig. 3. Photomicrograph (× 30) of a transverse section of the proventriculus, showing the anterior part of the cathaemasid inside the enlarged gland. Observe the atrophy of the glandular epithelium in comparison with the normal gland lying by its side.

atrophy, the acini being cystically dilated; and the lamina propria is infiltrated with lymphocytes, plasma cells, heterophiles and eosinophiles. Evidently this stage of the parasite, after reaching the gland, remains in it for some time, causing pathogenic effects and increasing in size, with a corresponding increase in the sucker ratio. After emergence from the proventriculus, this stage travels towards the oesophagus on its way to the pharynx, which is its seat of predilection. A few specimens recovered from the oesophagus show, in addition to a further increase in the size of the body and a corresponding greater size of its organs, the beginning of the appearance of eggs in the uterine coils.

Echinochasmus sp.

A large number of specimens, all adults, were recovered from the small intestines of the painted storks available for examination during March, 1959. The intestines of all the infected birds showed characteristic nodules, nearly the size of a pea and hard to the touch, which protruded from the intestinal wall. Each nodule harboured

up to five flukes. The posterior portions of each fluke hung into the intestinal lumen, the major part of the body being contained inside the nodules, in which there were characteristic tunnels. The specimens clearly belong to the genus *Echinochasmus*, because the body is elongated and attenuated posteriorly and a reniform collar is present which has twenty-two collar spines arranged in a single row, the dorsal ones with a median chasm, and on each side two corner spines situated a little behind the terminal spine of the series of the row (Fig. 4). Species

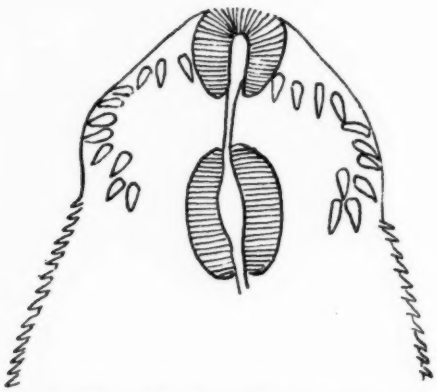


Fig. 4. Anterior part of the echinochasmid showing the reniform collar with twenty-two spines.

Fig. 5. Photomicrograph (× 35) of a transverse section of the small intestine, showing the echinochasmid lying inside the tunnel. Observe the necrosis and cellular infiltration around the fluke. The spinose integument of the parasite, its bifurcating intestinal caeca, the bipartite seminal vesicle, the acetabulum, the uterine coils, the ovary, both the testes and the vitellaria can be clearly made out.

of this genus found in India have so far been studied by Chatterji (1954), Gupta (1953), Lal (1939), Mudaliar (1938) and Verma (1935).

Two of the nodules just described were fixed and subsequently serially sectioned and stained. A study of these reveals that the flukes make tunnels in the submucosal and muscular coats of the intestinal wall, leaving the serous coat to form the tunnel's outer boundary (Fig. 5). The cavity of the tortuous tunnel occasionally shows here and there ova, detritus and bacterial flora and is, as stated above, in free communication with intestinal lumen. The cavity of the tunnel is bounded by a homogenous zone with sparse nuclei, which is apparently an area of caseous necrosis. External to this is a prominent belt of cellular infiltration consisting of plasma cells, lymphocytes, heterophiles and eosinophiles and the whole mass is encapsulated by collagenous fibrous tissue.

Travassos (1938) has described, in infections of *Euxenura maguri* and *E. galeatus* with *Choanocephalus penduriformis* in Brazil, a cystic formation with two flukes inside each cyst.

The authors are grateful to Sri C. V. G. Choudary, Principal of this College, for the facilities provided and to Dr H. D. Srivastava, Head of the Parasitology Division, I.V.R.I., for the loan of some literature.

REFERENCES*

Bhalerao, G. D. (1926). The trematodes of *Corvus insolens* (a Burmese house crow) with a description of four new species. *Parasitology*, **18**, 387–98.

Chatterji, P. N. (1954). On a new species of *Echinochasmus* from the intestine of a dog. *Indian J. Helminth.* **6** (1–2), 1–6.

Gupta, R. (1953). Studies on trematode parasites of Indian birds. I. On *Echinochasmus antigonus* n.sp. from sarus crane, *Antigone antigone* Linn. *Indian J. Helminth.* **5** (2), 81–6.

Lal, M. B. (1939). Studies in Helminthology. Trematode parasites of birds. *Proc. Indian Acad. Sci.* **10**, 111–200.

Mudaliar, S. V. (1938). A description of the trematode *Echinochasmus narayani* n. sp. with a comparison of some of the known species of the genus. *Indian Vet. J.* **15** (2), 109–14.

Travassos, L. (1938). Informacões sobre a fauna helminthologica de Matto Grosso. *Mem. Inst. Osw. Cruz*, **33** (4), 461–8.

Travassos, L. (1951). O' genero *Pulchrosoma* Travassos, 1916 e sua situacão no sistema de trematodeos. *Arq. Zool. Estado S. Paulo*, **7**, 465–92.

Verma, S. C. (1935). Studies on the Indian species of the genus *Echinochasmus* Pt. I and on an allied new genus *Episthochasmus*. *Proc. Indian Acad. Sci.* **1** (12), 837–56.

Yamaguti, S. (1958). *Systema Helminthum*, Vol. 1. *The Digenetic Trematodes of Vertebrates*, Parts I and II. New York, London: Interscience Publishers Inc.

* Only references relating to two genera studied have been included. The papers published on other species are listed in full in the Bibliography in *Systema Helminthum*.

Parasitology (1960), **50**, 329–348
With 1 *plate and* 11 *text-figures*
Printed in Great Britain

The physiology of the second ecdysis of parasitic nematodes

By W. P. ROGERS
University of Adelaide, South Australia
and R. I. SOMMERVILLE
McMaster Laboratory, C.S.I.R.O., Parramatta Road, Glebe, N.S.W., Australia

(*Received* 20 *July* 1959)

CONTENTS

I. Introduction 329
II. Materials and methods 330
III. Procedure and results 332
Studies on the sheath 332
(*a*) Changes during exsheathment 332
(*b*) The effect of osmotic pressure on the sheath 332
(*c*) The measurement of changes in the sheath 333
The stimulus for exsheathment 334
(*a*) The instability of stimulating substances 334
(*b*) Activity *in vivo* and *in vitro* 334
(*c*) The effect of hydrogen-ion concentration on activity of rumen fluid *in vitro* 335
(*d*) The effect of the oxidation-reduction potential 335
(*e*) The effect of temperature 338
The effects of stimulating larvae 338
(*a*) Evidence for a trigger mechanism 338
(*b*) Time of release of exsheathing fluid 340
(*c*) Exsheathing fluid activity in homogenates 340
(*d*) Location of the exsheathing mechanism in larvae 341
(*e*) The effect of inhibitors on exsheathment of *Trichostrongylus axei* . . 343
Properties of exsheathing fluid 343
(*a*) The relationship of nitrogen content to activity 343
(*b*) The effect of dialysis 343
(*c*) The effect of temperature 344
(*d*) Specificity of exsheathing fluid 344
(*e*) The effect of inhibitors 344
IV. Discussion 345
V. Summary 347
References 347

I. INTRODUCTION

Sommerville (1957) and Rogers & Sommerville (1957) examined the process of exsheathment of third-stage trichostrongyle larvae. They found that the process took place in two stages. First the host provided a stimulus which caused the larvae to secrete an 'exsheathing fluid' and, secondly, the exsheathing fluid attacked parts of the sheaths so that the larvae, aided by their own movements, were able to escape from the sheath.

Rogers (1958) found that similar mechanisms were concerned in the hatching of eggs of *Ascaris lumbricoides* and was able to give some biochemical details of the two stages. The stimulus for hatching the eggs depended upon the concentration of dissolved carbon dioxide, the pH and the oxidation-reduction potential of the medium. This caused the secretion within the egg of a 'hatching fluid' which contained a chitinase, esterase and possibly a proteinase. The hatching fluid attacked the shell of the egg thus releasing the infective stage.

In this paper a detailed description of the two processes concerned in the exsheathment of several species of trichostrongyle larvae is given as a preliminary study to the examination of the biochemistry of these processes.

II. MATERIALS AND METHODS

Infective larvae were obtained from sheep with pure infections of one of the following species: *Haemonchus contortus*, *Ostertagia circumcincta*, *Trichostrongylus axei* and *T. colubriformis*. Faeces from these sheep were incubated at 27° C. in glass jars, from which infective larvae were collected after 7 days and stored at 2° C. until required. Larvae were commonly used between 1 and 3 weeks after collection.

Six sheep with permanent rumen fistulae were used. They were fed *ad lib.* a mixture of wheat, wheaten chaff and lucerne chaff. Larvae were exsheathed *in vivo* by placing them in a cellophane sac in the rumen for 2 hr.

For experiments *in vitro*, larvae were exsheathed in a preparation of rumen fluid which had first been filtered and freeze-dried. The solid was dissolved in phosphate buffer pH 6, 0·067 M, and the pH adjusted with hydrochloric acid if necessary. Larvae and a reducing agent, sodium dithionite, at the rate of 1 mg./ml. were added, the preparation was sealed with liquid paraffin and incubated at 38° C.

Criterion of exsheathment. In most experiments larvae were classified as exsheathed if the refractile ring (Lapage, 1935; Sommerville, 1957) had commenced to form. Usually 100 larvae were counted and exsheathed larvae expressed as a percentage of this number.

Dissection and ligation of larvae. For critical work, in which larvae were severed at a constant distance from the anterior end, larvae were anaesthetized in an aqueous solution of ether and dissected under a microscope on a slide marked with two lines at the appropriate interval. Larvae were ligatured with nylon thread 12·5μ in diameter. A loop was made in the thread, the larva was guided through the thread for the required distance and the thread pulled tight. Larvae ligatured in this manner survived for at least 24 hr.

Preparation of exsheathing fluid. This has been described by Sommerville (1957). When stored at 1° C. activity was retained for at least 2 weeks. Before use the pH was adjusted to 7·5 with hydrochloric acid.

'Sheath-free' exsheathing fluid was prepared in the same way as normal exsheathing fluid, except that the sheaths were first removed by brief immersion of larvae in 5% sodium hypochlorite (Lapage, 1935). Inactivated exsheathing fluid was prepared by addition of mercuric chloride to a concentration of 0·003 M.

Estimation of the activity of exsheathing fluid. Empty sheaths with the anterior end intact were obtained by making an incision about the middle of the larva, which then burst out of the sheath. Sheaths of approximately uniform length were selected for estimation of activity because exsheathing fluid was found to attack short sheaths more quickly than long sheaths. Sheaths, in groups of 5, were incubated at 38° C. in hanging-drop preparations of exsheathing fluid. At intervals the preparation was examined and activity assessed, using a scoring method described in a later section.

Preparation of homogenates. About three million infective larvae were homogenized at 2° C. with glass powder and phosphate buffer, pH 6·5, 0·067 M. The homogenate was finally centrifuged at 0° C. for 10 min. and the supernatant fluid collected.

Preparation and tests of exsheathing fluid antiserum. Exsheathing fluid antiserum was prepared by injecting *H. contortus* exsheathing fluid, 1 ml. at a time, into a rabbit at intervals of 48 hr. for 14 days. One week elapsed before the animal was bled.

To study precipitate formation, larvae were immersed in antiserum mounted on a slide under a cover-slip; excess antiserum was removed to restrict movement. Antibody-antigen reactions were examined in Ouchterlony plates prepared from 0·7% agar to which had been added 'Merthiolate' at a concentration of 1:10000.

Preparation of metabolic products. Two million *H. contortus* larvae were suspended in a 5% solution of sodium hypochlorite until the sheaths were removed. Larvae were then washed several times in sterile water and finally suspended in 25 ml. of sterile water containing penicillin (500 units/ml.) and streptomycin sulphate (5 mg./ml.). A few larvae were observed occasionally to exsheath in water and this was prevented by keeping the temperature at 20° C. and by passing air through the suspension. After 72 hr. the larvae were removed and the solution examined for bacterial contamination. Culture on Wright's agar showed that there were less than 5 organisms per ml.

Apparatus for ultraviolet irradiation. A microscope was set up with an ultraviolet source. Light from the source was passed through a slit and focused in the plane of the object by a reversed reflecting lens in place of the usual substage condenser. Two slits which gave images 15 and 60 μ wide respectively were used. *T. axei* larvae were first anaesthetized in an aqueous solution of ether and then mounted on a quartz slide under a cover-slip. While the microscope and the image were focused, an ultraviolet filter was held between the source of light and object. The filter was then withdrawn and the object irradiated for 2–3 min.

Measurement of hydrogen-ion concentration and oxidation-reduction potential. Hydrogen-ion concentrations were measured with a glass electrode. Oxidation-reduction potentials were measured potentiometrically with a saturated calomel half-cell. Potentials were checked before and after each experiment with quinhydrone in phthalate buffer at pH 4 and in 0·1 N-hydrochloric acid. Errors at these potentials were never more than 5% and were usually less than 2%.

III. PROCEDURE AND RESULTS

Studies on the sheath

(*a*) *Changes during exsheathment.* The changes which exsheathing fluid induced in the sheath of larvae of *T. axei* and which lead to fracture of the sheath were examined. The following description, based on *T. axei*, is also applicable to *H. contortus* and *T. colubriformis*.

Exsheathing fluid first acted on a small area encircling the cuticle 19 μ from the anterior end, where it formed a 'refractile ring' (Lapage, 1935). The site of this ring was marked in some specimens of *T. axei* by a faint line (Sommerville, 1957) but this was not seen in either *H. contortus* or *T. colubriformis*.

The appearance of larvae in the early stages of exsheathment is shown in Pl. 1, fig. 1. The sheath often fractured immediately the refractile ring was formed and allowed the larvae to escape. If, however, the sheath failed to fracture, a second refractile ring was often formed about 60 μ from the anterior end. At the same time, the anterior end of the sheath became distended and often split longitudinally into two layers. Commonly this did not extend to the part of the sheath in front of the refractile ring, known as the 'cap', neither did it extend more than 60 μ from the anterior end (Pl. 1, fig. 2). Occasionally the cap also was included and the entire end of the sheath presented a swollen appearance with the remains of the inner layer twisted about the larva. In some sheaths small additional refractile rings formed anterior to the principal ring, but they did not play a part in the fracture of the sheath, which invariably broke at the principal refractile ring.

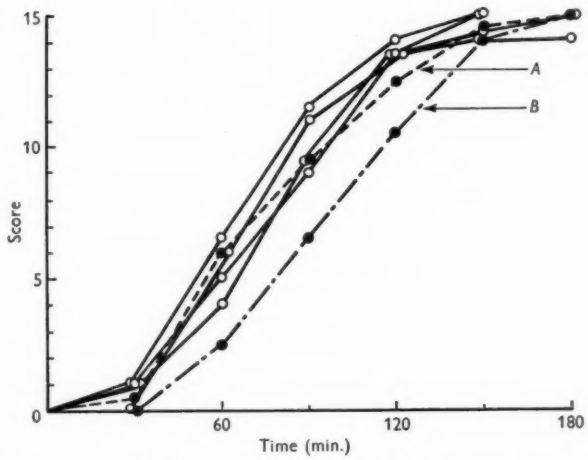
In the initial stage of formation of the refractile ring, the innermost layer of the cuticle became thinner, but, at least in the early stages, was not broken (Pl. 1, fig. 3). The sheath appeared to split into two distinct layers and the outer layers of the sheath about the refractile ring were often swollen. A small cavity was formed, which eventually led to longitudinal splitting of the sheath.

The regions of the sheath which reacted visibly with exsheathing fluid were small, for although additional refractile rings sometimes formed in front or behind the first, elsewhere the sheath was not affected. Empty sheaths which were ligated before immersion in exsheathing fluid were also unaffected.

(*b*) *The effect of osmotic pressure on the sheath.* When larva with swollen and distended sheaths (Pl. 1, fig. 2) were placed in 0·46 M sodium chloride the sheath collapsed, but it expanded again when the larvae were placed in water. Similarly, when empty sheaths were filled with 0·46 M sodium chloride, ligated and placed in distilled water, they became turgid, although after 1·5–3 min. they became flaccid again. Evidently the water passed through the sheath rapidly and the sodium and chloride ions more slowly. This implied that the openings of the excretory duct and anus in the sheath were no longer patent, which was confirmed by direct observation. The cuticle which lined the excretory pore and anus was withdrawn from the larva and crumpled against the side of the sheath, forming a seal. Therefore any substances, including exsheathing fluid, which were released by the larva passed into the space between the larva and the sheath, where they accumulated or diffused through the sheath.

(c) *The measurement of changes in the sheath.* When sheaths were dissected from larvae and placed in exsheathing fluid, the changes were less than those which were seen in normal intact sheaths. The anterior end of the sheath did not swell, only one refractile ring was formed and the 'cap' did not become detached. However, the formation of the refractile ring itself was much the same *in vitro* and *in vivo*.

The formation of the refractile ring took place in three stages. These were: stage 1, a small notch appeared (Pl. 1, fig. 4A); stage 2, the notch extended more than half the thickness of the sheath (Pl. 1, fig. 4B); stage 3, the sheath split longitudinally (Pl. 1, fig. 4C). Towards the end of stage 2, or more commonly in stage 3, the empty sheaths often collapsed along the dorsal and ventral lines and obscured details of the refractile ring. It was therefore occasionally necessary to roll the sheath over and re-examine it.



Text-fig. 1. Assessment of activity of exsheathing fluid. Each line represents mean of two determinations, each of which was made on five sheaths. For further explanation, see text.

The curves in Text-fig. 1 were obtained from twelve hanging drops made from the same preparation of *H. contortus* fluid. Each drop contained five sheaths of *H. contortus* and the hanging drops were incubated in pairs. At intervals the sheaths were examined and allotted a score of 1, 2 or 3 according to the stage they had reached. The mean total scores for each pair of hanging drops were then plotted. Curve *B* was displaced relative to the others. This curve and curve *A*, as a representative of the remainder, were converted to probits and the displacement was found to be significant ($P < 0{\cdot}05$), but there was no significant difference between the slopes ($P > 0{\cdot}5$). Displacement of curve *B* could be expected if the start of the reaction was delayed, such as was observed when long sheaths were used.

The stimulus for exsheathment

(*a*) *The instability of stimulating substances.* The activity of different samples of rumen fluid in promoting the exsheathment of *T. axei* varied greatly, and active samples were often inactivated by mild treatment such as centrifuging, filtering or freeze-drying. Boiling also caused inactivation.

In some experiments with *T. axei*, activity could be restored by adding a reducing agent, sodium dithionite 1 mg./ml., to rumen fluid under a layer of paraffin. This procedure, as shown in Table 1, consistently failed to restore activity for the exsheathment of *H. contortus*: with *T. axei*, however, the results varied from 5 to 100% reactivation with different samples of rumen fluid. With a given sample of rumen fluid, the results were always consistent (Table 1). These results are worth considering because they show that at least two unstable factors, one of which was probably a reducing system, were needed for stimulating *T. axei* and that the requirements for *H. contortus* were more rigorous.

Table 1. *The effect of boiling, aeration and reducing agent upon exsheathing properties of rumen fluid. The results were obtained in duplicate experiments,* (*a*) *and* (*b*), *with one lot of rumen fluid*

Treatment of rumen fluid	Percentage exsheathed			
	T. axei		*H. contortus*	
	(*a*)	(*b*)	(*a*)	(*b*)
Unboiled	74	80	58	40
Unboiled and reducing agent	82	88	38	58
After boiling	0	2	2	2
After boiling and reducing agent	72	61	16	6
After aeration	26	21	7	3
After aeration and reducing agent	62	82	15	2

(*b*) *Activity* in vivo *and* in vitro. The proportion of larvae of *T. axei* which exsheathed after different periods of exposure to rumen fluid *in vitro* and *in vivo* is shown in Text-fig. 2.

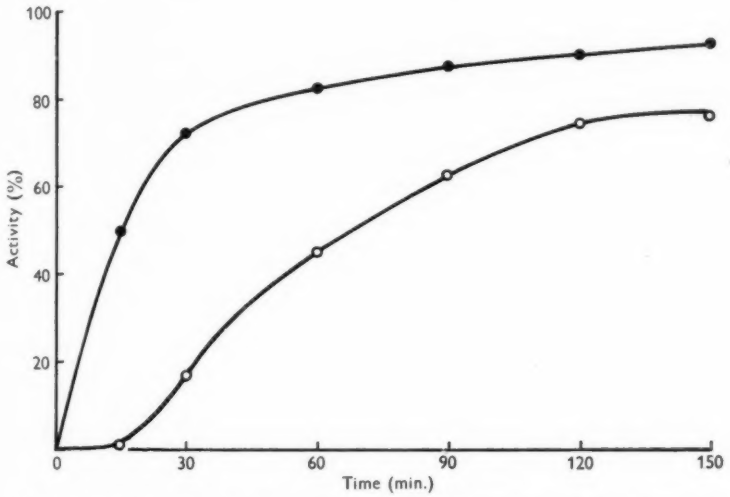
The rumen fluid sample was filtered, the pH adjusted to that of the rumen (6·5) and sodium dithionite and a layer of liquid paraffin added. The preparation was then incubated with larvae at 38° C. At the same time a cellophane sac, which contained a suspension of larvae of *T. axei* in water, was placed in the rumen of the sheep.

The results showed that under some conditions exsheathment could be obtained *in vitro* at rates comparable with those observed *in vivo*. Similar results were obtained with rumen fluid which had been freeze-dried and dialysed. The activity of this preparation was also enhanced by addition of sodium dithionite and a layer of liquid paraffin.

The relationship between activity of freeze-dried dialysate of rumen fluid and its nitrogen content was examined. Freeze-dried material was made up at different concentrations in M/100 phosphate buffer at pH 6·8; no further adjustment of the

pH was needed. After adding larvae and sodium dithionite, 1 mg./ml., the preparation was sealed with liquid paraffin and incubated at 38° C. for 4 hr. (*T. axei*) or 6 hr. (*H. contortus*). The longer period of incubation was used because *H. contortus* exsheathed more slowly than *T. axei* under these circumstances.

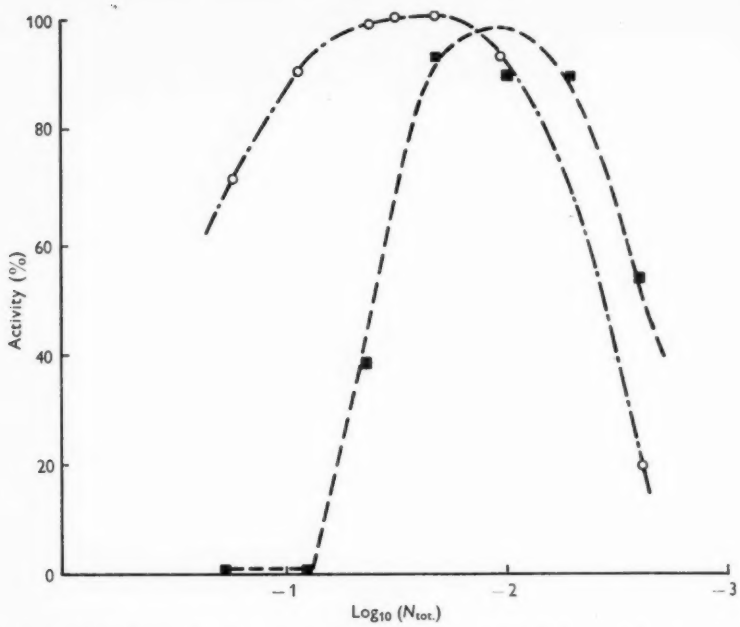
Typical results are shown in Text-fig. 3. The nitrogen content of the freeze-dried material varied from 0·5 to 0·8 %, on a dry-weight basis in the four preparations which were examined. Salt presumably formed a considerable proportion of the freeze-dried dialysate, and the osmotic pressure of solutions which contained as much as 20 mg. of total solids per ml. must have been high. This may have decreased the exsheathing activity at high concentrations of nitrogen. Indeed, larvae which had been subjected to high concentrations of freeze-dried material were shrivelled and distorted. Moreover, the activity at lower concentrations of nitrogen was sharply reduced by adding sodium chloride at concentrations of more than 25 mg./ml.



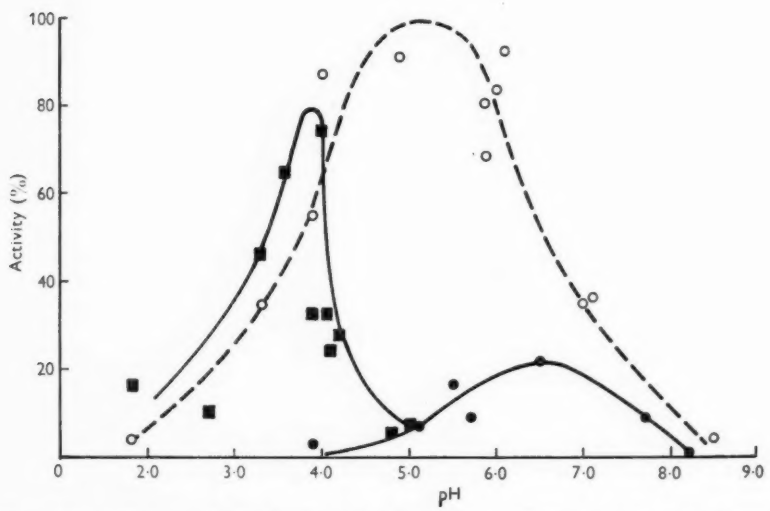
Text-fig. 2. Exsheathment of *T. axei* in a cellophane sac in the rumen compared with exsheathment in rumen fluid *in vitro*. ●, *in vivo*; ○, *in vitro*. $Na_2S_2O_4$, 1 mg./ml., and a layer of liquid paraffin were added to the rumen fluid.

(c) *The effect of hydrogen-ion concentration on activity of rumen fluid* in vitro. The effect of hydrogen-ion concentration on the exsheathment of *T. axei*, *T. colubriformis* and *H. contortus* in a preparation of freeze-dried rumen fluid is shown in Text-fig. 4. Duplicate samples were incubated for 120 min. and each point represents the mean of two determinations.

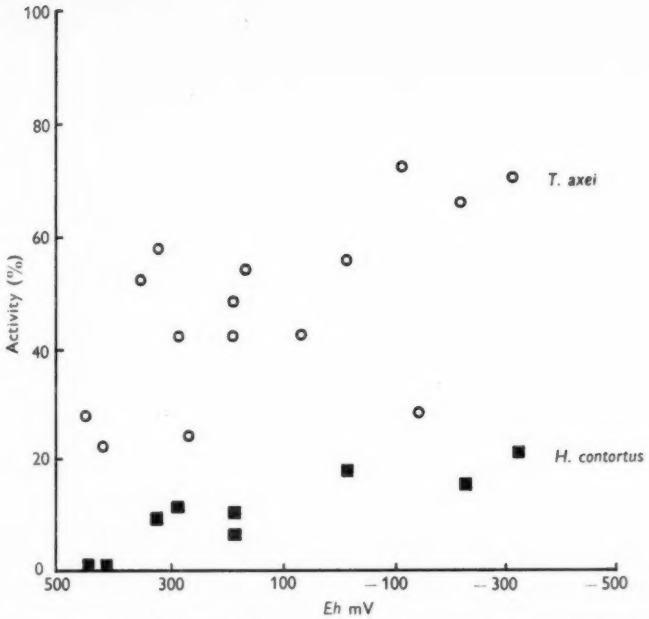
(d) *The effect of the oxidation-reduction potential.* The effect of sodium dithionite on the activity of rumen fluid suggested that activation might be affected by oxidation-reduction potentials. This was tested by incubating larvae in cultures of *Escherichia coli* which had been incubated for different periods and so reached different oxidation-reduction potentials. When suitable potentials had been reached, the larvae were added and the pH and potentials determined. After



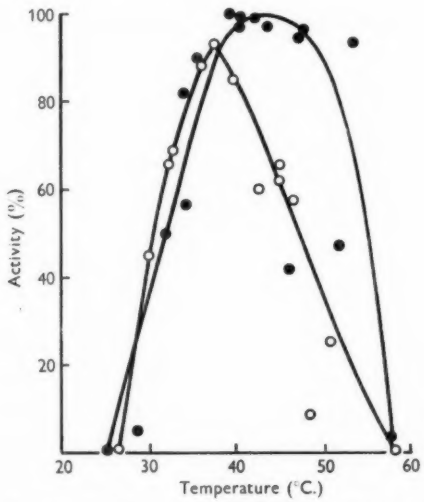
Text-fig. 3. Relationship between activity of freeze-dried dialysate of rumen fluid and its nitrogen content (mg./ml.). ○, *T. axei*; ■, *H. contortus*.



Text-fig. 4. Relationship between hydrogen-ion concentration and exsheathment in rumen fluid *in vitro*. ○, *T. axei*; ●, *H. contortus*; ■, *T. colubriformis*. Larvae were incubated in presence of $Na_2S_2O_4$, 1 mg./ml.



Text-fig. 5. Effect of oxidation-reduction potential on exsheathment of *T. axei* and *H. contortus*.

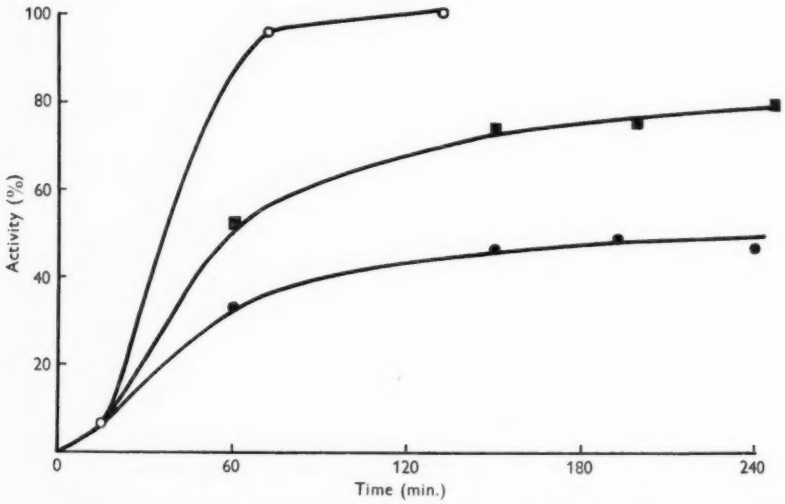


Text-fig. 6. Effect of temperature on exsheathment of *T. axei*. ○, Incubated 45 min.; ●, incubated 120 min.

2 hr. readings were taken again and the proportion of exsheathed larvae was measured. The pH in these experiments ranged from 6·5 to 6·8. In very young and very old cultures the potentials ranged from about 200 to 400 mV. At other times the potential fell as low as −350 mV. Generally, the activity of the culture in promoting exsheathment increased as the potential fell (Text-fig. 5). There was considerable variation in the results, however, and it seems clear that factors other than the oxidation-reduction potential affected the larvae.

(*e*) *The effect of temperature.* Larvae of *T. axei* were incubated in freeze-dried rumen fluid in phosphate buffer at pH 6. Reducing agent was added and the preparations sealed with liquid paraffin. The results obtained with incubation times of 45 and 120 min. are given in Text-fig. 6.

At temperatures between 50 and 60° C. larvae were still, but they continued to exsheath. The longer period of incubation increased the proportion of exsheathed larvae at higher temperatures, but it had little effect at lower temperatures. No larvae which had been heated at 60° C. exsheathed.



Text-fig. 7. The trigger mechanism in *O. circumcincta*. ○, Incubated in rumen fluid throughout; ■, incubated in rumen fluid 15 min., then water at 38° C.; ●, incubated in rumen fluid 15 min., then water at 14° C.

The effects of stimulating larvae

(*a*) *Evidence for a trigger mechanism.* If larvae were briefly exposed to stimuli which induced exsheathment and were then washed and incubated in water the process of exsheathment continued. Results from an experiment with *O. circumcincta* are shown in Text-fig. 7. It seems that incubation for 15 min. at 38° C. in rumen fluid was sufficient to trigger the process of exsheathment, which continued in the absence of the rumen fluid in a large proportion of the larvae. Similar results were obtained with larvae of *T. axei*.

One explanation of these results would be that the rumen fluid stimulated larvae to produce an exsheathing fluid which then acted on the sheath independently of the presence of the rumen fluid. This trigger mechanism offered a means for the study of the effects of reducing agent, hydrogen-ion concentration and temperature on the stimulus and on the process of exsheathment after larvae had been stimulated.

The effect of a reducing agent was studied with larvae of *T. axei*. The larvae were divided in two groups and stimulated in a preparation of freeze-dried rumen fluid at 38° C. One of these preparations contained sodium dithionite, 1 mg./ml., and was sealed with liquid paraffin; the other was aerated. After 15 min. the larvae were washed in water and incubated, as shown in Table 2 A, with a reducing agent or aerated.

Table 2. *The effect of aeration, temperature and hydrogen-ion concentration on the trigger mechanism*

A. Effect of aeration

Trigger 15 min.	Water 2 hr.
Aerated: 0% exsheathed	Aerated: 0% exsheathed Reduced: 0% exsheathed
Reduced: 44% exsheathed	Aerated: 82% exsheathed Reduced: 98% exsheathed

B. Effect of temperature

Trigger 10 min.	Water 2 hr.
38° C.: 0% exsheathed	38° C.: 71% exsheathed 14° C.: 32% exsheathed
14° C.: 0% exsheathed	38° C.: 0% exsheathed 14° C.: 0% exsheathed

C. Effect of pH

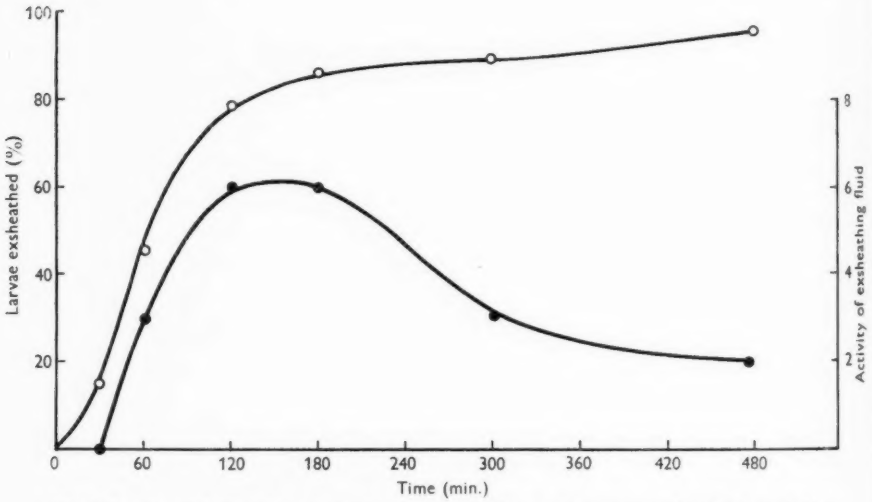
Trigger 10 min.	Water 2 hr.
pH 6: 20% exsheathed	pH 6: 88% exsheathed pH 10: 100% exsheathed
pH 10: 0% exsheathed	pH 6: 40% exsheathed pH 10: 0% exsheathed

Similar experiments were carried out to examine the effect of temperature and hydrogen-ion concentration on the larvae during the period of stimulation and during the later process of exsheathment. The results are given in Table 2, B and C.

It appears that the presence of a reducing agent was necessary during the period of stimulation but not during the subsequent process of exsheathment. Larvae failed to exsheath when stimulation was attempted at 14° C., irrespective of subsequent treatment. After stimulation at 38° C., they exsheathed more slowly on subsequent incubation at 14° C. than at 38° C. The effect of hydrogen-ion concentration during the period of stimulation was pronounced; a relatively high concentration of hydrogen ion was necessary to obtain activity, although subsequently the pH of the external medium did not appear to be important.

(*b*) *Time of release of exsheathing fluid.* Exsheathing fluid was detected when larvae of *T. axei* were incubated in a cellophane sac in the rumen of a sheep, but it was not detected when the same larvae were subsequently incubated as soon as they were removed from the rumen fluid.

Further experiments were then made to follow the production of exsheathing fluid in the presence of rumen fluid. Larvae in a cellophane sac were placed in the rumen of a sheep and samples of the enclosed fluid and larvae collected at intervals. Larvae were examined for exsheathment and the fluid for exsheathing activity (Text-fig. 8). As the proportion of exsheathed larvae increased, the concentration of exsheathing fluid also rose, but after 3 hr. the concentration began to fall. The activity of exsheathing fluid was greatest at the time when the largest number of larvae were exsheathed and commenced to fall after nearly 90 % of larvae had exsheathed. Larvae which were exsheathed in the rumen, then removed and returned 30 min. later, again produced exsheathing fluid. This could be repeated a third time.



Text-fig. 8. Relationships between the percentage of larvae exsheathed and the concentration of exsheathing fluid in the suspension. ○, Percentage of *T. axei* exsheathed; ●, activity of exsheathing fluid.

It appeared, therefore, that larvae could be stimulated to release exsheathing fluid for a brief interval only. Further release of exsheathing fluid was induced only if the stimuli were temporarily withdrawn.

(*c*) *Exsheathing fluid activity in homogenates.* When infective larvae of either *T. axei* or *H. contortus* were homogenized with powdered glass in phosphate buffer at pH 6·5, the supernatant obtained after centrifugation showed activity similar to exsheathing fluid. Comparable results were obtained from homogenates of exsheathed larvae.

Homogenates from *H. contortus* were highly active against sheaths of *T. axei* as well as against sheaths of *H. contortus*, but homogenates of *T. axei* showed little

or no activity against sheaths of *H. contortus*. Dilute solutions of exsheathing fluid showed similar specificity. In addition, active supernatant from homogenates of *T. axei* had no effect on sheaths from infective larvae of *Nippostrongylus muris*, and homogenates of *N. muris* larvae were active against sheaths of *N. muris* but not against those of *T. axei*. The specificity and behaviour of these homogenates indicated the presence of exsheathing fluid in the larvae.

(*d*) *Location of the exsheathing mechanism in larvae.* Sommerville (1957) showed that in infective larvae of *T. axei*, cells between the excretory pore and the base of the oesophagus were involved in the process of exsheathment, but his results did not preclude the possibility that cells in more posterior regions were also concerned. Therefore larvae which had been ligated immediately behind the base of the oesophagus were severed on the posterior side of the ligature. These larvae were able to exsheath, and it was concluded that the exsheathing mechanism did not extend behind the base of the oesophagus.

The role of sensory structures on the head end of the larvae was examined in similar experiments made on the anterior end. It was not possible to do this without destroying the region of the cuticle which reacted with exsheathing fluid, and to detect the presence of exsheathing fluid it was necessary to incubate the larvae in small volumes of fluid in the presence of empty sheaths. A suitable vessel of about 0·35 μl. capacity was made by burning a small depression in 'Perspex'. Preliminary tests showed that not only could larvae exsheath when sealed in this vessel under liquid paraffin with reducing agent and rumen fluid, but also exsheathing fluid was detected by the formation of refractile rings in empty sheaths which were incubated with the larvae. Each experiment was made with 40–50 infective larvae which had first been freed from their second-stage sheaths with sodium hypochlorite.

Approximately 50 infective larvae of *T. axei* were severed about 70μ from the anterior end and the posterior portions incubated in the vessel with empty sheaths, water and a small bubble of air. These larvae released exsheathing fluid, whereas none was detected when intact larvae were used. Therefore exsheathing fluid was not released by the normal mechanism. Either cells in which the fluid was stored were damaged, or damage to any cells released substances with activity similar to exsheathing fluid.

To test this hypothesis, larvae were severed about the mid-point and the posterior portions incubated in both water and rumen fluid, but exsheathing fluid was not detected. Similar experiments were made with the anterior ends of larvae (60μ or 70μ), but again these failed to produce any exsheathing fluid. It therefore seems probable that when larvae were severed about 70μ from the anterior end, the injury was sufficiently extensive to damage cells in which exsheathing fluid was held. These cells were apparently more than 70μ from the anterior end but did not extend past the base of the oesophagus.

It was noticed that the posterior end of the oesophagus and the associated cells spilled out of the severed larvae, but when larvae were ligatured immediately anterior to the excretory pore and severed in front of the ligature, the oesophagus was retained in place. Larvae so treated failed to release exsheathing fluid when

incubated with water in the microcell but did so when incubated under a seal with reducing agent and extracts of rumen fluid.

These experiments suggested that not only was exsheathing fluid stored in the region of the larvae between the base of the oesophagus and the excretory pore, but also that receptors for the stimulus to exsheath as well as the mechanism for release of exsheathing fluid lay in this region.

Ultraviolet radiation. Seventeen infective larvae of *T. axei* were each exposed for 2 or 3 min. to a narrow beam of ultraviolet light. The light passed through a slit which gave an image 60μ wide and this enabled the irradiated area to be restricted to the region between the base of the oesophagus and the excretory pore. All these larvae failed to exsheath when incubated under liquid paraffin in rumen fluid with sodium dithionite. A similar number of larvae were irradiated in the mid-body region about 300μ from the anterior end, and half of these exsheathed when incubated in rumen fluid. Similar results were obtained when other parts of larvae were irradiated. Exsheathment was therefore inhibited only when ultraviolet light was directed on the region between the base of the oesophagus and the excretory pore. However, irradiation throughout this region with a beam of light 15μ wide did not prevent exsheathment.

Antibody-antigen reactions. Antiserum to exsheathing fluid of *H. contortus* was collected from a rabbit and overlaid with exsheathing fluid. The presence of antibodies was demonstrated by the formation of a precipitate at the interface. Precipitates were not observed when antiserum was overlaid with either physiological saline or a dialysate of rumen fluid; neither were they observed when normal serum was overlaid with exsheathing fluid.

When larvae which had been freed from their sheaths by sodium hypochlorite were stimulated to produce exsheathing fluid in the sheep's rumen and then bathed in exsheathing fluid antiserum, precipitates formed at the excretory pore of most larvae and nowhere else. Precipitates formed readily at 2° and 38° C., and they also formed on larvae which had not been stimulated. Thus, though the larvae produced antigenic substances, it was not clear that exsheathing fluid itself was antigenic.

Ouchterlony agar-gel diffusion plates were used in an attempt to determine the nature of the antigenic components of larval excretions. The following antigens were used: normal exsheathing fluid; exsheathing fluid which had been inactivated by the addition of mercuric chloride to a final concentration of 10^{-3}M; 'sheath-free' exsheathing fluid, prepared from larvae which had first lost their sheaths in sodium hypochlorite; metabolic products. Exsheathing fluid antiserum was used as a source of antibody and plates were incubated at 30° C.

At least three bands (Pl. 1, fig. 5) formed in the reaction between 'sheath-free' exsheathing fluid and antiserum. The most intense band was also formed by normal exsheathing fluid, but its absence from inactivated exsheathing fluid suggested that it represented the active component of exsheathing fluid. A second and fainter band which lay close to 'sheath-free' exsheathing fluid could not be traced any further. However, the third band was common to normal, 'sheath-free' and inactivated exsheathing fluid. Several other bands which were absent from the 'sheath-free' system but present in the reaction with normal exsheathing

fluid presumably represented break-down products of the sheath. No bands were seen between metabolic products and exsheathing fluid antiserum although subsequent work showed that it was possible to get a weak antiserum by injection of metabolic products into a rabbit.

These data suggested that when 'sheath-free' larvae were activated at least three antigenic systems were produced, and it seems likely that the active component of exsheathing fluid was one of these systems.

Further attempts were made to identify the active components using neutralization techniques. It was found that exsheathing fluid was inactivated by the addition of diluted antiserum, but this inhibition was non-specific, for it was also observed when normal sera were used. However, antiserum which had been incubated at 30° C. for 5 hr. in the presence of air and hypochlorite-exsheathed larvae formed precipitates about the excretory pores of larvae only when these had first been stimulated in rumen fluid. This suggested that these precipitates were a result of a reaction between the active component of exsheathing fluid and its homologous antibody.

These experiments showed that the excretory pore was probably the site from which larvae release exsheathing fluid.

(*e*) *The effect of inhibitors on exsheathment of* Trichostrongylus axei. Potassium cyanide, sodium azide, iodoacetic acid, 2,4-dinitrophenol, sodium thioglycollate and copper sulphate at concentrations of 10^{-3} M had no consistent effect on the exsheathment of larvae of *T. axei* in rumen fluid with reducing agent at pH 5·8 –6·0.

Properties of exsheathing fluid

(*a*) *The relationship of nitrogen content to activity.* Exsheathing fluid of *H. contortus*, of known nitrogen content and at pH 7·5, was diluted to varying degrees with phosphate buffer pH 7·5. Exsheathing activity was then examined at the different concentrations of nitrogen. In Text-fig. 9 the relative activity obtained by incubating isolated sheaths for 120 min. at 38° C. in fluid of different dilutions is shown in relation to the logarithm of the nitrogen concentration in mg./ml.

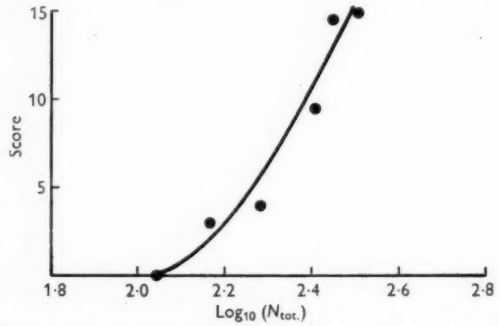
Attempts to concentrate exsheathing fluid by freeze-drying or by distillation under reduced pressure at 1° C. failed because activity was destroyed.

(*b*) *The effect of dialysis.* Exsheathing fluid of *H. contortus* was dialysed for 72 hr. against three changes of distilled water at 4° C. During dialysis the volume increased from 4·0 to 4·2 ml. The first change of water was freeze-dried and the residue taken up in 2 ml. of distilled water. Activity of exsheathing fluid was greatly reduced by dialysis (Text-fig. 10) and the dialysable fraction was inactive. However, the addition of this fraction to an equal volume of the dialysed exsheathing fluid restored activity. This experiment was repeated several times with similar results. In one of these experiments it was found that if dialysed exsheathing fluid was heated to 60° C. for 10 min. the addition of the dialysable fraction no longer restored activity. On the other hand, the ability of the dialysable fraction to restore activity was not impaired by heating.

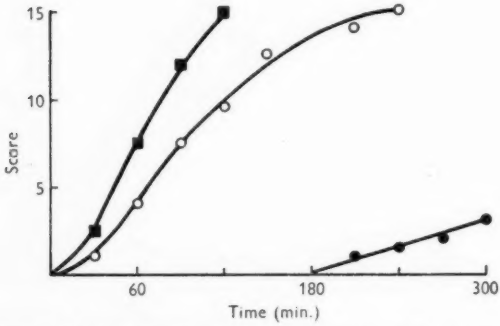
Activity of dialysed exsheathing fluid was also restored by adding either magnesium chloride or manganous chloride to a concentration of 0·001 M.

(*c*) *The effect of temperature.* Exsheathing fluid of *H. contortus* was inactivated by heating at 60° C. for 10 min.; it showed activity at 1° C.

The effect of temperature on activity was measured over periods of 80 min. in hanging drops each of which held five sheaths. The results are shown in Text-fig. 11. The values for Q_{10}, calculated over the temperature range 29·5–39·5° C., varied from 1·9 in stage 2 of the process of exsheathment to 1·3 for stage 3.



Text-fig. 9. Relationship between activity of *H. contortus* exsheathing fluid and its nitrogen content (mg./ml.).



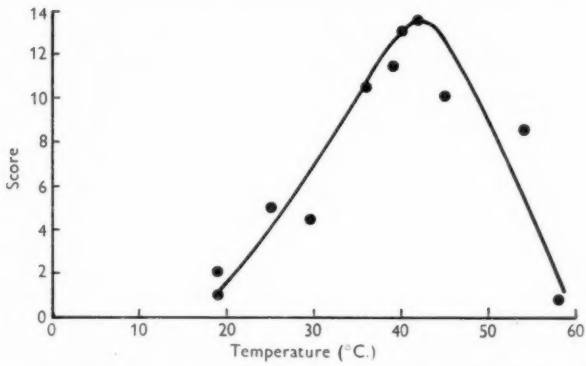
Text-fig. 10. Effect of dialysis on *H. contortus* exsheathing fluid. ■, Activity before dialysis; ●, activity after dialysis; ○, activity of dialysed exsheathing fluid after addition of dialysable fraction.

(*d*) *Specificity of exsheathing fluid.* The action of exsheathing fluid from *H. contortus* on its own sheaths was compared to its action on those dissected from *T. axei*, *T. colubriformis* and *Oesophagostomum columbianum*. After incubating for 78 min. at 38° C. it was found that the sheaths of *H. contortus* and *T. axei* were attacked to about the same degree. Changes in sheaths of *T. colubriformis* and *O. columbianum* had not proceeded very far.

Exsheathing fluid from *T. axei*, though it attacked sheaths from the same species quite actively, had little action on sheaths of *T. colubriformis*, *H. contortus* or *O. columbianum*.

(*e*) *The effect of inhibitors.* The exsheathing action of fluid from *H. contortus* was completely inhibited by Hg^{2+} (0·001 M) and was partly inhibited by Fe^{3+}

(0·001 M) and iodoacetic acid (0·05 M). Fe^{2+}, Ag^{2+}, and Pb^{2+} had no effect in concentrations up to 0·001 M. Ethylene diaminetetra-acetic acid (0·001 M), cysteine (0·01 M), glutathione (0·05 M), oxygenation and trypsin were also without effect.



Text-fig. 11. Relation between temperature and activity of *H. contortus* exsheathing fluid. Readings obtained after incubation for 80 min.

IV. DISCUSSION

The process of exsheathment may be considered in two stages: the extrinsic stage in which larvae are stimulated or activated by an external system in the host's alimentary tract, and an intrinsic phase in which activated larvae secrete an exsheathing fluid which attacks the sheath.

The process was influenced by the hydrogen-ion concentration, oxidation-reduction potential and temperature of the environment. The trigger mechanism made it possible to evaluate the significance of these environmental factors. For example, it was clear that both hydrogen-ion concentration and oxidation-reduction potential of the external environment were of special significance in the process of activation. Larvae which had been triggered subsequently exsheathed at hydrogen-ion concentrations and oxidation-reduction potentials outside those values which permitted activation. Similarly, although temperatures above 25° C. were necessary for activation, exsheathment continued at lower temperatures, although more slowly than at 38° C. It is not clear from these experiments, however, whether the importance of these physical states lay in their effect on the larva, either as part of the 'activator' or by conditioning the larva to respond to some activator, or whether the hypothetical activator was transformed into an active state by specific physical conditions.

In the rumen, both *H. contortus* and *T. axei* commonly exsheath at about the same rate (Sommerville, 1957). However, in *in vitro* experiments, despite controlled oxidation-reduction potentials, hydrogen-ion concentration and temperature, *T. axei* occasionally and *H. contortus* commonly showed little exsheathment. There may therefore be important variables which, although present in the rumen, were incompletely controlled in *in vitro* experiments with rumen fluid. The results

in Table 1 suggest that these variables were adversely affected by boiling and by aeration and that *H. contortus* was more critical in its requirements than *T. axei*.

The experiments with ligated larvae showed that neither the cells posterior to the base of the oesophagus, nor sensory organs about the anterior end, such as the amphids, were necessary for exsheathment. It was not possible to exclude the nerve ring and its associated ganglia, however, because the ligature, although invariably in front of the excretory pore, was not always in front of the nerve ring. It seemed likely, therefore, that the entire mechanism for reception of the stimulus to exsheath, for accumulation of exsheathing fluid and for its release, lay between the base of the oesophagus and the level of the excretory pore or the nerve ring. This conclusion was confirmed by the failure of larvae to exsheath when irradiated with a beam of ultraviolet light 60μ wide. Failure of the narrow (15μ) beam of ultraviolet light to inhibit exsheathment suggested that the irradiation damaged a system which extended over the greater part of the region between the excretory pore and the base of the oesophagus.

It has been suggested, although without direct evidence, that the excretory pore is the site from which exsheathing fluid is released (Sommerville, 1957; Rogers & Sommerville, 1957). The excretory pore is not only the sole orifice in the region between the nerve ring and the base of the oesophagus, but also it discharges into the space between the outer sheath of the infective larva and the inner, third-stage sheath. Additional evidence which pointed to the excretory pore as a likely point for release of exsheathing fluid, was provided by the antigen-antibody reactions. Activated larvae discharged antigenic material only from the excretory pore, and although direct evidence was lacking, it seemed probable that exsheathing fluid was antigenic and was one of the components of the precipitate about the excretory pore.

The presence of exsheathing fluid in homogenates prepared from sheathed larvae suggested that larvae accumulated exsheathing fluid, and that this was released when larvae were activated. The process of activation appeared to be a stimulus for the release of pre-formed exsheathing fluid and to have no direct effect on exsheathing fluid itself. This conclusion is supported by the observation that larvae which had been severed at any point along the oesophagus released exsheathing fluid, in spite of the absence of conditions which might lead to activation. Apparently the injury enabled exsheathing fluid to leak out of a cell or cells in which it was stored. Unfortunately, the anatomy of these cells is not known in sufficient detail to enable any storage cells to be identified.

The reserve of exsheathing fluid was evidently not exhausted by initial activation and exsheathment, for not only did exsheathed larvae release further fluid when stimulated a second and a third time, but exsheathing fluid was also detected in homogenates of exsheathed larvae.

Exsheathing fluid used in these studies was prepared as a very dilute and impure solution and its activity measured by a semi-quantitative method. In spite of these disadvantages it has been possible to learn something of its properties. The effect of temperature on activity, together with the inhibition by Hg^{2+} and Fe^{3+}, suggested that exsheathing fluid included a protein. A low molecular weight

cofactor was present which was heat-stable and necessary for activity. The cofactor was replacable by Mn^{2+} and Mg^{2+} and it could be that the cofactor was indeed one of these two ions, for the chelating agent, ethylene diaminetetra-acetic acid, has a relatively low affinity for Mg^{2+} and Mn^{2+} (Mellor & Maley, 1948).

V. SUMMARY

When third-stage trichostrongyle larvae exsheathed, they released an exsheathing fluid which eroded the sheath at the anterior end. This led to the formation of a refractile ring and ultimately to the fracture of the sheath which enabled the larva to escape.

Exsheathment was induced by ovine rumen fluid. The exsheathing activity of rumen fluid was impaired by centrifuging, filtering, boiling and aeration, but activity could be restored by the addition of reducing agent. The activity *in vitro* of either fresh rumen fluid or freeze-dried rumen fluid, after addition of a reducing agent, was comparable with that obtained *in vivo*.

After stimulation, the process of exsheathment continued in the absence of rumen fluid. The ability of rumen fluid to stimulate larvae was dependent upon temperature, hydrogen-ion concentration and oxidation-reduction potential.

Exsheathing fluid was present in larvae both before and after exsheathment. Larvae which had been stimulated to exsheath released exsheathing fluid for a brief interval and further release could be obtained only after the stimuli had been temporarily withdrawn.

The mechanisms for reception of the stimulus to exsheath and for storing and releasing exsheathing fluid were located in a region between the base of the oesophagus and the excretory pore. The fluid was probably released from the excretory pore.

Exsheathing fluid from one species was not necessarily as active against sheaths from other species as against its own. The activity of exsheathing fluid was dependent upon the presence of a heat-stable cofactor of low molecular weight. Activity of the dialysed fluid could be restored by addition of $MnCl_2$ or $MgCl_2$. Values of Q_{10} for the action of the fluid on the sheath lay between 1·3 and 1·9. Activity was inhibited by Hg^{2+}, Fe^{3+} and iodoacetic acid.

We are indebted to Mrs M. Ross, University of Adelaide, and Mr B. M. Wagland, C.S.I.R.O. McMaster Laboratory for technical assistance. Mr B. Thompson, McMaster Laboratory, supplied the larvae, and the photographs were taken by Mr I. T. Roper, McMaster Laboratory.

One of us (W. P. R.) wishes to acknowledge a grant from the George Aitken Pastoral Research Trust.

REFERENCES

Lapage, G. (1935). The second ecdysis of nematode infective larvae. *Parasitology*, **27**, 186–206.

Mellor, D. P. & Maley, L. (1948). Order of stability of metal complexes. *Nature, Lond.*, **161**, 436–7.

Rogers, W. P. (1958). Physiology of the hatching of eggs of *Ascaris lumbricoides*. *Nature, Lond.*, **181**, 1410–11.

ROGERS, W. P. & SOMERVILLE, R. I. (1957). Physiology of exsheathment in nematodes and its relation to parasitism. *Nature, Lond.*, **179**, 619–21.

SOMMERVILLE, R. I. (1957). The exsheathing mechanism of nematode infective larvae. *Exp. Parasitol.* **6**, 18–30.

EXPLANATION OF PLATE 1

Fig. 1. Refractile ring formation in an infective larvae of *T. axei*.

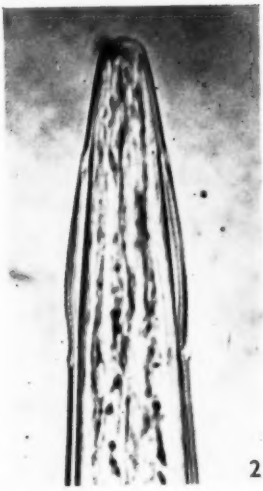
Fig. 2. Infective larva of *T. axei* which failed to exsheath when the refractile ring first formed. The sheath has swollen behind the refractile ring and is commencing to split longitudinally. A second refractile ring has formed at the posterior (lower) end of swelling.

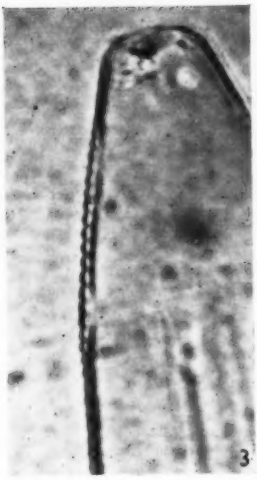
Fig. 3. Refractile ring formation in *T. axei*. The inner layer of the sheath has been attacked by exsheathing fluid and is visible as two parallel lines.

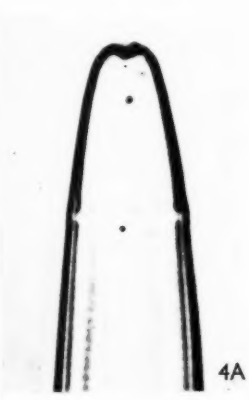
Fig. 4. Sheaths of *T. axei* in hanging-drop preparations of rumen fluid. A, stage 1; B, stage 2; C, stage 3.

Fig. 5. Reaction between (*A*) exsheathing fluid antiserum; (*B*) inactivated exsheathing fluid; (*C*) normal exsheathing fluid; (*D*) 'sheath-free' exsheathing fluid; (*E*) metabolic products of infective larva.

1

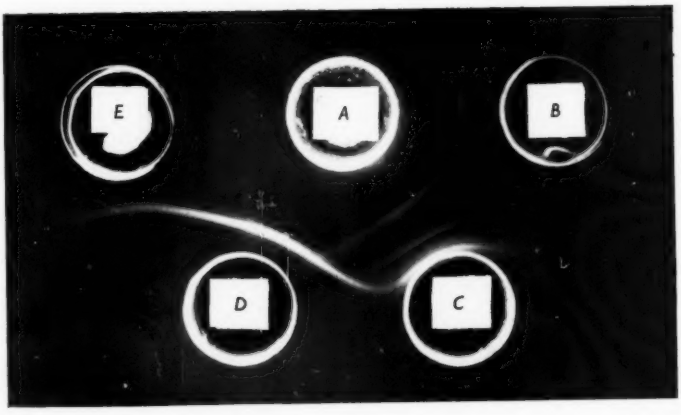

2


3


4A

4B

4C


E
A
B
D
C

Parasitology (1960), **50**, 349–350
Printed in Great Britain

The problem of Mehlis's gland in helminths with special reference to *Penetrocephalus ganapatii* (Cestoda: Pseudophyllidea)

By K. HANUMANTHA-RAO, D.Sc.*

Department of Zoology, University of Leeds, and *The Molteno Institute, University of Cambridge*

(*Received* 28 *July* 1959)

In a recent paper Hanumantha-Rao (1960) has endeavoured to give a preliminary account of the histochemistry and the possible functions of Mehlis's gland in the pseudophyllidean cestode *Penetrocephalus ganapatii*. Since communicating the paper efforts were directed to analyse histochemically the problem of Mehlis's gland in the liver fluke, *Fasciola hepatica*, by means of rigorously controlled procedures and to determine if the secretion is a phospholipid in this case also. It became apparent that with every phospholipid test Mehlis's gland in *F. hepatica* proved positive. Moreover, negative results with the same tests in parallel sections after extraction with a fat solvent such as pyridine have been consistent.

MEHLIS'S GLAND AND EGG-SHELL FORMATION IN *PENETROCEPHALUS GANAPATII*

The failure of Mehlis's gland in *Penetrocephalus ganapatii* to react to phospholipid stains such as Baker's acid haematein and Sudan black B is now thought to be due solely to the fact that material fixed in Bouin's fluid was used, whereas material fixed in formaldehyde-calcium followed by post-chromation is essential for these tests. There appears to be some difference of opinion with regard to the periodic acid-Schiff reaction. Casselman (1959) has shown, in a tabular statement, that the periodic acid-Schiff positive reaction is a feature of glycolipids and not phospholipids. Mehlis's gland secretion is positive to all the phospholipid stains and is also strongly positive to the periodic acid-Schiff technique. This may mean that glycolipids may also be associated with the secretion but, because evidence from the Nile-blue test is completely lacking, some other explanation must be sought to explain the periodic acid-Schiff reactivity. Pearse (1953) lists phosphatides under periodic acid-Schiff positive substances, and unsaturated fatty acids are known to be periodic acid-Schiff positive (Wolman 1950). If, as suggested by Hanumantha-Rao (1959), it is considered that the phospholipid secreted by Mehlis's gland is in fact lecithin then the problem would appear to have been solved, because one of the fatty acid radicals in lecithin is an unsaturated one.

It was stated that Mehlis's gland secretion may be incorporated into the vitelline cells during their transit through the ootype. However, the secretion apparently

* Permanent address: Department of Zoology, Andhra University, Waltair, India.

absorbed by the vitelline cells remains periodic acid-Schiff negative until the cells have released all shell material. When the egg-shell is constructed the vitelline cells in the egg become strongly periodic acid-Schiff positive and tend to give the same reactions as Mehlis's gland. In the work on *Fasciola hepatica* it was suggested that the lecithin-like phospholipid may be enzymatically converted into an agent capable of effecting the release of egg-shell precursors and the possible similarity of this process to haemolysis was pointed out. In *Penetrocephalus ganapatii* exactly the same situation could be envisaged. So in the lysolecithin state, which presumably is the agent mentioned above, lecithin would have lost the unsaturated fatty acid radical thus making the secretion periodic acid-Schiff negative. This is why, beyond the Mehlis's gland region, there was not a positive periodic acid-Schiff reaction, either in the ootype or in the vitelline cells during transit. After the egg-shell precursors have been extruded and the egg-shell is constructed, the general function of Mehlis's gland may be said to have been fulfilled. Later it appears that by re-esterification of lysolecithin it is converted back to lecithin which forms the food yolk and thus the vitelline cells become strongly periodic acid-Schiff positive.

It is, however, recognized that at the present state of our knowledge this explanation about what may be considered as one of the most formidable problems in helminth physiology, necessarily appears over-simplified. At the same time it becomes clear that some ideas of the histochemistry and functions of Mehlis's gland are emerging.

SUMMARY

1. Mehlis's gland secretion in trematodes and pseudophyllidean cestodes may be a lecithin-like phospholipid.

2. After enzymic degradation of the lecithin into lysolecithin the release of egg-shell precursors from the vitelline cells is effected.

3. Soon after the completion of the egg-shell the lysolecithin may be reconverted into lecithin in vitelline cells of *Penetrocephalus ganapatii*, thus accounting for the periodic acid-Schiff positive reaction.

My thanks are due to Colombo Plan authorities for a scholarship, and to Professor E. A. Spaul for facilities and help. It is with great pleasure that I record my grateful thanks to Dr P. Tate for his help and advice.

REFERENCES

CASSELMAN, W. G. B. (1959). *Histochemical technique*, London: Methuen.

HANUMANTHA-RAO, K. (1959). Histochemistry of Mehlis' gland and egg-shell formation in the liver fluke *Fasciola hepatica* L. *Experientia*, **15**, 464.

HANUMANTHA-RAO, K. (1960). Studies on *Penetrocephalus ganapatii*, a new genus (Cestoda: Pseudophyllidea) from the marine teleost *Saurida tumbil* (Bloch). *Parasitology*. **50**, 155–162.

PEARSE, A. G. E. (1953). *Histochemistry*, J. & A. Churchill Ltd.

WOLMAN, M. (1950). Staining of lipids by periodic acid-Schiff reaction. *Proc. soc. Exp. Biol.* **75**, 583–85.

Parasitology (1960), **50**, 351–363
With 5 text-figures
Printed in Great Britain

Herpetomonas ludwigi (Kramář, 1950) n.comb., the trypanosomatid parasite of cranefly larvae (Diptera, Tipulidae)

By KEITH VICKERMAN*
Department of Zoology, University of Exeter

(*Received 6 October*, 1959)

CONTENTS

		PAGE
I.	Introduction	351
II.	Materials and methods	352
	(1) The host	352
	(2) Collection of larvae	352
	(3) Identification of larvae	352
	(4) Examination of larvae	352
	(5) Microscopical methods	352
III.	Observations on the flagellate	353
	(1) Incidence and distribution	353
	(2) Living material	354
	(3) Leptomonad forms	354
	(4) Attached or haptomonad forms	357
	(5) Crithidial forms	357
	(6) Trypaniform forms	358
	(7) Precystic forms	358
	(8) Cysts	358
	(9) Division stages	358
IV.	Discussion	360
	(1) Life cycle	360
	(2) Taxonomic position	361
	(3) Relationship with other species of *Herpetomonas*	361
V.	Summary	362
	References	363

I. INTRODUCTION

Whilst providing one problem for the agriculturist as a troublesome pest of cereal crops and grassland, cranefly larvae, or 'leatherjackets' as they are popularly termed, can provide several for the protozoologist, for the number of parasites to which these larvae are prone is prodigious. Larvae of species of *Tipula* from habitats other than grassland are also heavily infected.

Trypanosomatid flagellates from tipulid larvae were first recorded by Ludwig (1946). Out of 1500 freshwater-dwelling larvae of *Tipula abdominalis*, only two had a midgut infection of what Ludwig believed to be a species of *Leptomonas* Kent. The flagellates were attached to the midgut epithelium by their flagella. Kramář (1950) in a study of the protozoan parasites of *T. maxima* larvae (terrestrial) described leptomonad forms from the mid-gut diverticula and named the

* Present address: Department of Zoology, University College London.

organism *Leptomonas ludwigi* n.sp., for he believed it to be identical with the flagellate seen by Ludwig. The morphology of the free leptomonad, an attached form and a so-called 'leishmanial form' was briefly described by this author.

In the course of a study of Protozoa from *Tipula paludosa* larvae in Devon, a trypanosomatid flagellate was encountered which I believe to represent Kramář's organism. Further stages in the life history of the flagellate, described here, I think make it quite clear that the organism is more properly assigned to the genus *Herpetomonas* Kent, hence the new combination.

II. MATERIALS AND METHODS

(1) *The host*

Tipula paludosa, the common cranefly, lays its eggs in early autumn and the first three larval instars are to be found in the soil until the new year. Fourth instar larvae are to be found from December to August; by the end of August all larvae have pupated. After a fortnight in the pupal condition the imago emerges. The adults are on the wing in late summer and autumn.

(2) *Collection of larvae*

Early tipulid instars are not easily extracted from the soil, in fact they can only be procured by elaborate flotation techniques. Fourth instars, however, are readily obtained by applying an emulsion of orthodichlorobenzene to the turf (Barnes, 1937). The emulsion used in this work is sold commercially as 'Jeyes' Leatherjacket exterminator', although it is merely a severe irritant and does not kill the larvae (Milne, Coggins & Laughlin, 1958). About 10 ml. of the exterminator were added to a 2-gallon bucketful of water and the stirred emulsion distributed over 2 square yards of ground. The larvae emerged within 5–15 min of applying the exterminator; they were collected and washed free of the irritant in tap or stream water.

(3) *Identification of larvae*

Larvae were identified as belonging to the *paludosa* group of species of *Tipula* on Chiswell's (1956) key. They were identified as *T. paludosa* itself by allowing some from each batch to complete their development and then checking the adult characters on the key given by Oldham (1928).

(4) *Examination of larvae*

A larva washed free of debris was seized by the head capsule with forceps and its posterior end cut off with forceps scissors just anterior to the anus. Immediately the whole gut was expelled from the cut end. The operation was performed on clean filter-paper which served to soak up the haemocoel fluid. The oesophagus was cut through and the gut transferred to a clean slide. The morphology of the gut is shown in Fig. 1.

(5) *Microscopical methods*

Gut smears were either examined in 0·6% saline for the living flagellate, or fixed in order that permanent preparations might be made. Maier's modification of Schaudinn's fluid was used for the fixation of wet smears (Minchin & Thomson,

1915): fixation time was 15 min. Staining was in Heidenhain's iron haematoxylin (12 hr. mordant, 12 hr. stain, followed by differentiation in saturated aqueous picric acid solution) or by Delamater's Azure A technique (Delamater, 1951). In the latter, smears were hydrolysed for 2 min. in cold N-hydrochloric acid and then in hot acid at 60° C. for 4 min. After washing in the cold acid for a few seconds and in distilled water, they were stained in 0·25 % Azure A reduced by the addition of thionyl chloride, 2 drops in 10 ml. stain. Six hours was found to be a satisfactory staining time, and a half-saturated solution of chromotrope 2R in 96 % alcohol for 1 min. was used as counterstain. The majority of smears were, however, fixed for 2 min. in methyl alcohol and stained in Giemsa diluted 1:1 with distilled water at pH 7·2.

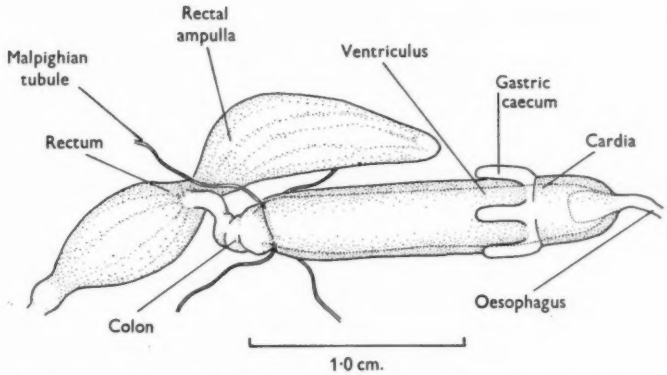


Fig. 1. Morphology of the gut of *Tipula paludosa*. The position of the peritrophic membrane is shown by a dotted line.

For sectioning, guts were fixed in Maier's fluid, cleared in cedar-wood oil and embedded in paraffin wax (m.p. 57° C.) before cutting at 10μ. It was found best to cover the sections with a film of celloidin after dewaxing, to prevent the rather heavy plant material in the gut leaving the sections during their passage through the alcohols, and so disrupting them. Sections were stained in iron haematoxylin as for smears, and differentiated in saturated aqueous picric acid.

III. OBSERVATIONS ON THE FLAGELLATE

(1) *Incidence and distribution*

The incidence of infection of larvae with *Herpetomonas ludwigi* in three populations examined is given in Table 1. In all cases the organism was confined to the enteric (gastric) caeca of the host.

This very low incidence could be artificially increased by two methods: (*a*) *overcrowding*. Runnage larvae kept in the laboratory for 2 months in crystallizing dishes packed with moss (*Acrocladium cuspidatum*) were found to be 100 % infected; cannibalism was rife among the larvae and this, combined with faecal contamination of the food, probably served to boost the infection rate; (*b*) *oxygenation of larvae*. Larvae maintained in pure oxygen at atmospheric

pressure showed a significant increase in the incidence of infection indicated in Table 2. A hundred Runnage larvae of the same batch but not subjected to oxygen were found to show only 6% infection when examined at the end of the experiment.

Table 1. *Incidence of* Herpetomonas *in larvae examined*

Locality of collection	No. larvae examined	No. infected	Infection (%)
Streamside pasture, Runnage, Dartmoor	200	12	6
Streamside pasture, Bellever, Dartmoor	100	5	5
Lawns of Hatherly Laboratories, Exeter	700	0	0

Table 2. *Effect of oxygen on incidence of infection in Runnage larvae*

Initial no. larvae	No. days in pure O_2	No. larvae surviving	No. infected	Infection (%)
20	3	20	3	15
20	4	18	5	27
20	5	14	2	14
20	6	16	6	38

In experimental animals, the flagellates, instead of being confined to the enteric caeca, were found throughout the peritrophic lumen of the mid-gut after oxygenation. This would seem to indicate that oxygen tension is important in restricting the distribution of the flagellate. Normally, owing to the fact that they are suspended in the haemocoel fluid, the oxygen tension in the caeca is presumably higher than in the rest of the mid-gut and the herpetomonads are only able to survive here. When the oxygen tension of the mid-gut is raised artificially the flagellates can invade its peritrophic lumen. The oxygen certainly makes for rapid multiplication of the flagellate and it is possible that in doing so it causes cryptic infections to become overt, hence the remarkable effect on incidence of infection.

(2) *Living material*

Fresh amounts of teased caecum showed bundles and rosettes of the flagellates. They were elongated flattened creatures which tended to be twisted spirally along the length of their bodies. The majority were immobile but some individuals now and then exhibited spasmodic locomotory movements accompanied by sigmoid flexures of the body. Leptomonad, haptomonad, crithidial, trypaniform and encysted stages were encountered, but the differences between some of these were not apparent until after fixation and staining. The morphology of each of these stages in the life cycle is described below.

(3) *Leptomonad forms*

These are characterized by the possession of a flagellum of varying length and having the kinetoplast situated nearer to the anterior end of the body than to the nucleus (Fig. 3A, B). The flagellates range in length of body from 8 to 26μ

and they are usually 1 or 2μ across at their widest point. The free flagellum of the leptomonad is up to 17μ long, but it rarely attains the body length. Movement of the flagellum appears to be confined to one plane and consists of a series of waves which diminish in amplitude as they pass along the flagellum towards its tip. In propelling itself through debris the organism appears to use its flagellum as a sensory probe or drill.

In the living leptomonad the nucleus and kinetoplast can be seen as shadowy structures in the finely granular and homogeneous cytoplasm. Sometimes larger refringent granules are present in the posterior portion of the animal. Lying immediately anterior to the kinetoplast is a clear area which has the appearance

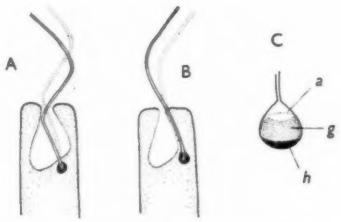


Fig. 2. Diagrams showing form of flagellar apparatus. A, Base of flagellum lies in reservoir; B, base of flagellum lies in wall of reservoir forming an intracytoplasmic axoneme; C, staining properties of the kinetoplast; *h*, haematoxylin and Delamater stains; *g*, Giemsa; *a*, achromatic zone.

of an anterior invagination or reservoir (Fig. 2A, B). On closely observing this area in an organism whose flagellum is rapidly moving, one occasionally sees that the intracellular part of the flagellum is also moving, that is, it is behaving as though it lay free in the liquid medium contained in the reservoir. In such cases the intracytoplasmic course of the flagellum, or axoneme, must be exceedingly short (Fig. 2A). In the majority of leptomonads, however, the base of the flagellum appears to be adherent to or embedded in the wall of the reservoir (Fig. 2B).

Giemsa-stained preparations of the leptomonad forms show a heavily stained nucleus, kinetoplast and flagellum. The kinetoplast is bean-shaped in outline; an achromatic zone separates it from the point of origin of the flagellum. No blepharoplast was observed with certainty in any preparation. In haematoxylin and Delamater preparations the kinetoplast is represented by a more flattened structure, presumably the deoxyribonucleic acid containing portion of the larger element stained by Giemsa's method (Fig. 2C). Although the relationships of structures on so small a scale are difficult to ascertain using light microscopy only, it might be added that the construction of the flagellar apparatus here reported well accords with that revealed for *Strigomonas oncopelti* with the aid of the electron microscope (Horne & Newton, 1958).

The nucleus is oval in shape and usually lies within the posterior two-thirds of the body, while posterior nucleated forms are quite common (Fig. 3B, C). On the whole Giemsa's stain gives worthless nuclear preparations, whereas haematoxylin staining of wet smears shows the nucleus as a vesicular structure containing

an ovoid, densely staining karyosome in a clear space surrounded by the nuclear membrane (Fig. 3O). Lying in this space about eight shadowy structures may be discerned, each closely applied to the nuclear membrane; these represent the chromatin elements of the interphase nucleus, which are stained much more clearly in preparations made by Delamater's method when they appear as conspicuous coarse lumps staining blue (Fig. 5A). If chromotrope 2R is used as counterstain,

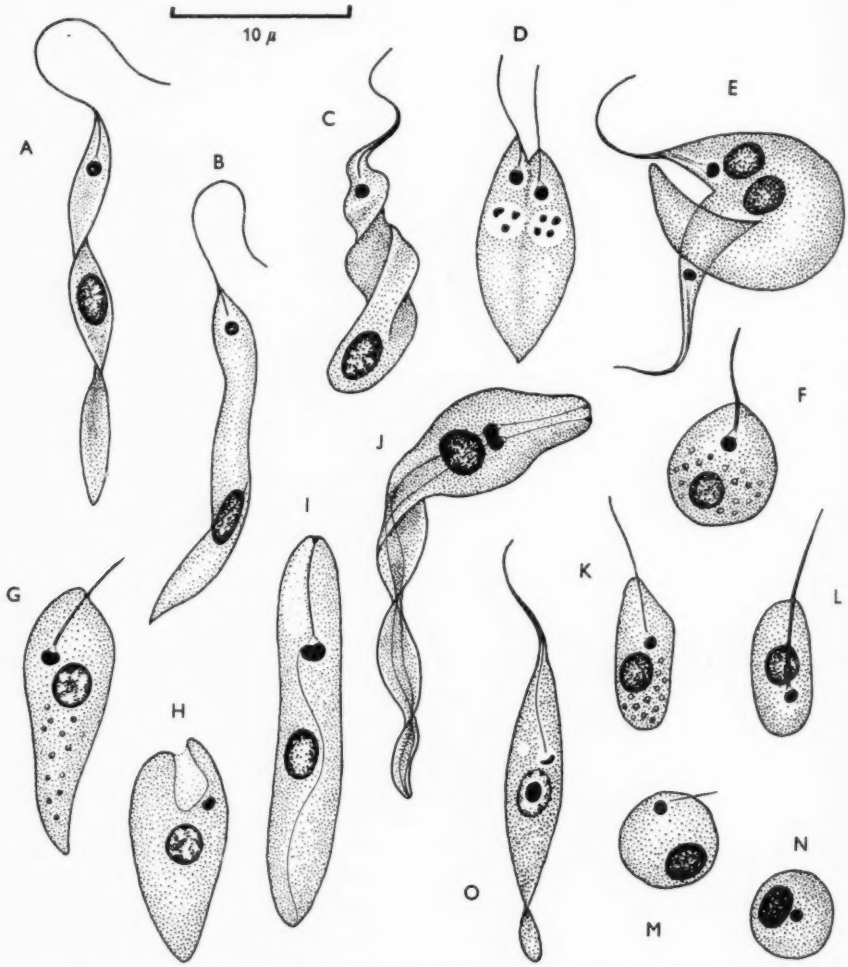


Fig. 3. *Herpetomonas ludwigi*. Camera lucida drawings of stages in life-cycle as seen in Giemsa and iron haematoxylin-stained preparations. A, B, C, leptomonad forms showing varying position of nucleus; D, E, dividing leptomonads; F, short leptomonad; G, H, I, J, haptomonad forms, G, with short flagellum, H lacking flagellum but with prominent reservoir, I and J with axoneme and axoplast; these structures double in J; K, L, short forms with kinetoplast in crithidial and trypaniform positions, respectively; M, N, precystic forms, all Giemsa stained; O, crithidial form stained in iron haematoxylin showing nuclear structure.

the karyosome may be coloured pink but it is usually obscured by the chromatin elements.

In Giemsa-stained preparations some extremely flattened flagellates exhibit a very fine axoplast running through the posterior part of the body. It appears to originate at the kinetoplast and to pursue a wavy course to the posterior tip of the flagellate (Fig. 3I, J). This structure has not been observed in either living flagellates or those stained by methods other than Giemsa. A diagrammatic representation of the structure of the leptomonad form as revealed by these techniques is shown in Fig. 4.

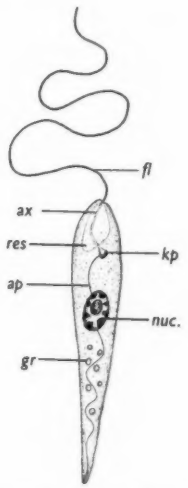


Fig. 4. Morphology of the leptomonad phase of *Herpetomonas ludwigi*, as revealed by a variety of techniques; *ax*, axoneme; *ap*, axoplast; *fl*, flagellum; *gr*, refringent granules of cytoplasm; *kp*, kinetoplast; *nuc*, nucleus; *res*, reservoir.

(4) *Attached or haptomonad forms*

The largest organisms observed in smears have only a minute remnant of a flagellum, or lack even that, and are non-motile. They measure up to 34μ in length and 3μ across. These will be referred to as haptomonad forms for they are in life found attached to the brush-border epithelium of the caecum. The exact mode of attachment is difficult to make out, but probably the short flagellum secures the organism; Ludwig suggested that it penetrates the host's cell. The haptomonad form is rounded or flattened anteriorly where it is applied to the gut wall, and it tapers posteriorly. The kinetoplast is placed as in the leptomonad or lies close to the nucleus. An axoneme is usually prominent but may be completely lacking, the latter condition frequently obtaining when the reservoir is well developed (Fig. 3H).

(5) *Crithidial forms*

In the crithidial form the kinetoplast is just anterior to the nucleus (Fig. 3O). Such forms are usually smaller than the leptomonads, 9–14μ in length, 1–2μ wide; they appear to arise as the result of a series of divisions in the later haptomonads

in which the kinetoplast has already approached the nucleus. The flagellum is still very short. In squashes of enteric caeca many, perhaps most, crithidial forms were attached. When dislodged they could be recognized by the lashing movements of the whole body anterior to the nucleus. An undulating membrane has never been observed in this or any other stage of the life history of the organism.

(6) *Trypaniform forms*

Some flagellates have the kinetoplast behind the nucleus, usually close to it but occasionally nearer to the posterior end of the body (Fig. 3L,; Fig. 5, D, H). These trypanosome-like forms are of similar size to the crithidia, or slightly smaller. The reservoir is absent from them and from many crithidia; a few small vacuoles visible at the anterior end may represent its remains (Fig. 4D). There is no evidence that these vacuoles are contractile (cf. Clark, 1959).

(7) *Precystic forms*

These are small round to pyriform flagellates in which the flagellum is short and the kinetoplast bears no particular relationship to the nucleus. The latter undergoes a reduction in size and, concomitantly, its chromatin becomes condensed to form a thick and irregular layer beneath the nuclear membrane (Fig. 5K). These nuclear changes are obvious in Delamater but not in Giemsa-stained preparations.

(8) *Cysts*

The cysts are represented by spherical leishmanial forms enclosed in a cyst-wall and measuring 3 or 4μ in diameter. The only obvious inclusions are the nucleus and the kinetoplast (Fig. 5L). The nucleus is now reduced to a small, solid ball of compacted chromatin. The kinetoplast lies so close to the nucleus that sometimes it cannot be distinguished as a separate body (Fig. 5M).

(*b*) *Division stages*

Division of the organism has been observed in leptomonad, haptomonad, crithidial and trypaniform stages. It appears to follow the pattern described by other workers for trypanosomatid flagellates, more especially that outlined by Sorouri (1957) for *Leishmania donovani*. The following account is taken from smears stained by the Delamater method. Unfortunately in these the karyosome was never well stained by the chromotrope 2R, although this has proved a very satisfactory counterstain for karyosomes in other flagellates and in amoebae. In most preparations staining of the karyosome was obscured by the densely staining chromosome material. Its precise role in mitosis is therefore not very clear.

Prophase is marked by the detachment of the six to eight chromatin bodies from the nuclear membrane (Fig. 5A, B). They migrate towards the karyosome. The chromatin bodies, called chromatids by Sorouri, then appear to pair; at any rate at metaphase only three or four bodies are discernible at the equatorial plate (Fig. 5C, E). At anaphase (Fig. 5F, H) the three or four chromosomes become

six or eight again as they diverge into two sets of daughter chromosomes, each set migrating towards a pole of the elongated karyosome. At telophase the karyosome presumably constricts into two, and each half together with one set of daughter chromosomes becomes enveloped by a portion of the nuclear membrane. Constriction of the karyosome has not been observed in Delamater preparations,

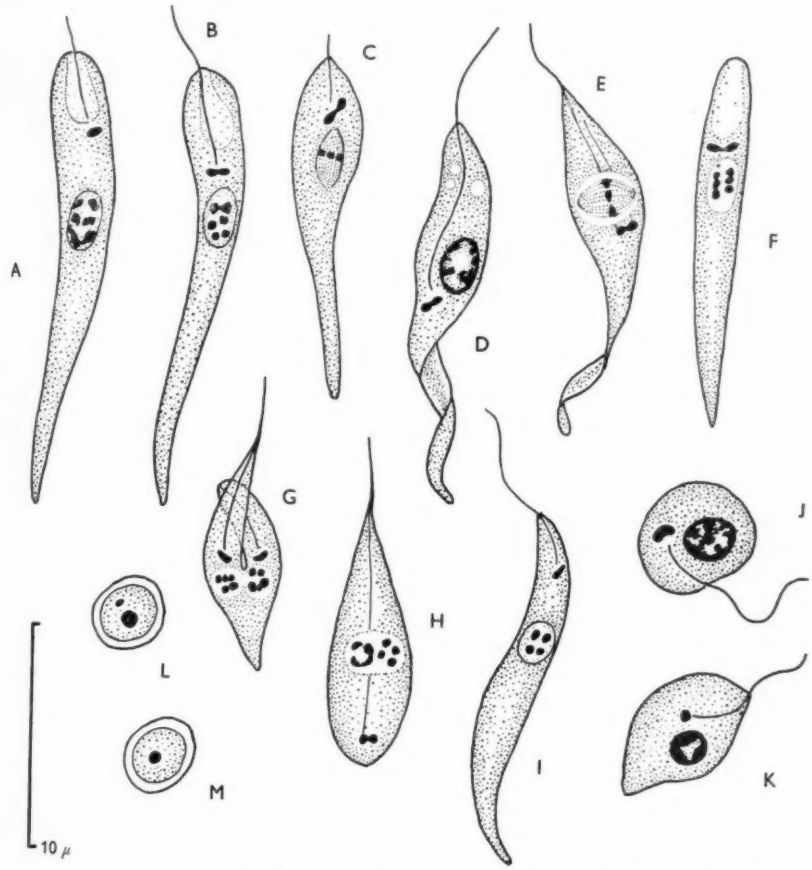


Fig. 5. *Herpetomonas ludwigi*. Camera lucida drawings of stages in life-cycle as seen in preparations stained by Delamater's method. A, B, haptomonad forms with nucleus in prophase; C, same with nucleus in metaphase; D, E, trypaniforms showing constriction of kinetoplast accompanying interphase and metaphase, respectively; F, advanced constriction of kinetoplast, nucleus in anaphase (?); G, crithidial stage in division, nucleus in telophase; H, anaphase in trypaniform stage; I, recently divided leptomonad; J, K, precystic forms; L, M, cysts.

only in haematoxylin ones. It is difficult to decide from preparations stained by either method whether or not the nuclear membrane persists throughout division. It is at telophase that the chromosomes are most easily seen and then they are usually four in number (Fig. 3D and Fig. 5G).

Meanwhile the kinetoplast has divided into two or is in the process of doing so. I have been unable to detect any strict relationship in time between the division of the kinetoplast and the division of the nucleus; the two events seem to take place independently of one another (Fig. 5B, C, G). Hence it seems improbable that the kinetoplast complex, or any part of it, has a centriolar function. Of the two daughter kinetoplasts one retains the rhizoplast and flagellum, the other develops these structures anew. The organism splits from its anterior end posteriorly between the two kinetoplasts and subsequently between the daughter nuclei. Throughout division the old flagellum continues to beat; the new flagellum has grown to over one-half its final length before it starts to beat.

IV. DISCUSSION

(1) *The life cycle*

From a study of the associations of the above forms in smears and from a study of sections of infected caeca, the life cycle in this species appears to be as follows. Concerning the earliest developmental stages of *Herpetomonas ludwigi* I am able to say little beyond speculation. Presumably the cyst hatches to produce a 'preflagellate' in the cardia of the larva and this gives rise to a motile organism which can make its way from the intraperitrophic lumen to the caecum. However, I have not seen any of these forms in the process of penetration through the peritrophic membrane in smears of cardia contents. It is probable, however, that the leptomonas stage (Fig. 3A, B, C) represents the migratory phase. It has already been noted that this form is very adept at boring its way through debris using its flagellum as a mechanical drill. This ability would enable the flagellum to penetrate through the peritrophic membrane (most probably at its origin where it is still soft) into the extraperitrophic space of the cardia. Leptomonads are never very numerous in the caeca and, although they have been seen in division, it seems likely that this phase is a short lived one and that migration is its chief function. Further evidence for this will be discussed later.

Once inside the caecum the leptomonas loses its flagellum, or the greater part of it, and becomes attached to the caecal wall. A growth phase then ensues, the haptomonads (Fig. 3G–J) being significantly larger than the leptomonads. The axoneme increases in length (at the expense of the free flagellum?) as the kinetoplast comes to lie next to the nucleus. The growth phase is followed by one of rapid multiplication to form many small crithidia (Fig. 3K). These forms thickly line the caecum; eventually the older ones wriggle out or are squeezed out into the caecal lumen. Here they are transformed into the trypaniform stages, with the kinetoplast behind the nucleus (Fig. 3L). These are short lived, and, although they may divide, they eventually round off to form the precystic flagellates. The nucleus shrinks, the chromatin becomes compacted, the organism itself shrinks in volume and the flagellum is resorbed. These precystic forms (Fig. 3M, N) correspond to Kramář's so called 'leishmanial' forms. Finally a cyst wall is laid down. In sections of the caecum the cysts can be seen occupying the centre of the lumen.

At intervals the caecum contracts, pumping out its contents into the peritrophic space of the mid-gut. On such occasions all unattached organisms will be ejected. Free swimming leptomonads are therefore in danger of being voided at each caecal contraction, hence this phase is short lived and throughout its growth and division stages the flagellate is attached. These contractions, however, provide an exit for the cysts into the mid-gut and hence into the hind-gut and out with the faeces. Infection of other larvae is by the ingestion of cysts or, as leatherjackets feed avidly upon one another, by cannibalism.

(2) *Taxonomic position*

The state of confusion in the nomenclature of the insect trypanosomatids stems from Saville Kent's (1881) inadequate descriptions. Wenyon (1913) and Hindle (1912) have discussed the problem at length. Briefly the position is this: Kent erected the genus *Leptomonas* for a flagellate, *L. bütschlii*, seen by Bütschli in the gut of the nematode *Trilobus gracilis*. In the same work—in fact two pages later—he created the genus *Herpetomonas* for the flagellate parasite of the house-fly originally described by Burnett (1851) as a species of *Bodo*, and later by Leidy as *Bodo muscarum*. Judging from Kent's descriptions *Herpetomonas* and *Leptomonas* are synonymous; however, since his time it has been found that *H. muscarum* has crithidial and trypaniform stages in its life cycle (Prowazek, 1904). *Leptomonas bütschlii*, the type-species of its genus, has not been restudied since its discoverer found it and so it is not known whether or not it possesses these developmental stages. Until it has been restudied the genus *Leptomonas* remains valid for all those trypanosomatid flagellates in which the kinetoplast at no time lies next to the nucleus.

Grassé (1952) does not recognize the genus *Herpetomonas*, for he believes that all species of *Leptomonas* (the genus which has paginal priority) have developmental stages as described for *Herpetomonas*, and that in the past the search for these has been insufficiently thorough. This is not so: for example, Gibbs (1951, 1954) has described several species of *Leptomonas* from South African bugs in which these stages do not occur. Hence I shall recognize the two genera as being distinct from one another and assign the organism under discussion to the genus *Herpetomonas*, making Kramář's species *H. ludwigi* n.comb.

(3) *Relationship with other species of* Herpetomonas

There is little doubt that this organism is the same as the one described by Kramář. The close relationship of the hosts (both terrestrial larvae of *Tipula* spp.), the confinement of the infection to the gastric caeca, together with the morphology and size range of the leptomonad and attached forms may be cited as supporting evidence. As *H. ludwigi* is a parasite of the larvae of non-biting flies, it may be asked if the organism is in actual fact identical with species from other such hosts. It is known from the work of Mackinnon (1910) that *H. muscarum* (Leidy) [= *H. muscae-domesticae* (Stein)] inhabits the gut of various larval dung flies (*Homalomya*?, *Scatophaga*, *Neuroctena*). As the tipulid flagellate was only found in larvae from pasture upon which cattle had been grazing, it was thought possible

that larvae might pick up the infection from cysts of *Herpetomonas muscarum* washed into the soil from dung in which dung-fly larvae were feeding: in other words the tipulids were harbouring *H. muscarum*. But a comparison of the life cycle and morphology of that organism with the one described here forbids acceptance of the synonymy.

Morphologically the principal difference between the two flagellates lies in the form of the kinetoplast. In *H. muscarum* this is elongated and rod-like and takes on the form of a 'Y' shape at the commencement of division. The kinetoplast of *H. ludwigi* is spherical to pyriform, never rod-like.

The second difference is a developmental one. As far as I can discover from the numerous authors on the life of *H. muscarum* (Wenyon, 1913, 1926; Becker, 1923) there is no long attached or haptomonad phase in that flagellate as there is in *H. ludwigi*. In the latter attachment has been shown to be of considerable adaptive significance. Since these flagellates are restricted to the enteric caeca, their complete development would be prevented if they were discharged into the midgut each time the caeca contracted. Some sort of anchorage is necessary throughout the growing and reproductive periods. On the other hand, in *H. muscarum* the flagellate is found throughout the whole gut; there is no restriction to any one region and consequently little need for anchorage except in the rectum, where the precystic flagellates may be attached. The leptomonad stage of *H. muscarum* is the form in which growth and division take place; divisions are so numerous that in smears the majority of the organisms have a 'double flagellum' (Prowazek, 1904). In *H. ludwigi* divisions are rare in the leptomonad form, as double flagella are not seen very often; the leptomonad stage here is short-lived. The case for a separate species for the *Herpetomonas* of tipulid larvae is therefore a reasonable one.

V. SUMMARY

1. *Herpetomonas ludwigi* was found in 5–6% of larvae of *Tipula paludosa* in two out of three populations examined.

2. The developmental cycle is confined to the enteric caeca of the host. From a study of smears and sections of these organs it was concluded that the life cycle was composed of the following stages:

(*a*) A long-flagellated leptomonad stage which is short-lived and probably represents the form in which the flagellate enters the caeca from the mid-gut lumen; (*b*) a haptomonad or attached form in which the flagellum is short or absent: this probably represents the growth phase of the organism; (*c*) a crithidial phase which arises by a series of divisions from the haptomonad; (*d*) a small trypaniform stage which breaks free and lives in the lumen of the caecum; (*e*) a precystic flagellate; and (*f*) a small, thick-walled cyst.

3. This species is characterized by its restriction to the enteric caeca and the short duration of free swimming as opposed to attached stages in the life history. This second feature is a corollary of the first. The periodic contraction of the enteric caeca makes attachment obligatory throughout the growth and division stages. These contractions also serve to void cysts into the mid-gut whence they leave the larva with its dejecta to infect other larvae.

I would like to thank Mr P. J. Dare for his help in collecting larvae from Dartmoor and Dr R. S. J. Hawes for much advice and criticism of the paper. The work was done during the tenure of an Agricultural Research Council Studentship.

REFERENCES

BARNES, H. F. (1937). Methods of investigating the bionomics of the common cranefly, *Tipula paludosa* Meigen, together with some results. *Ann. Appl. Biol.* **24**, 356.

BECKER, E. R. (1923). Observations on the morphology and life history of *Herpetomonas muscae-domesticae* in North American muscoid flies. *J. Parasitol.* **9**, 199.

BURNETT, W. I. (1851). The organic relations of some of the infusoria, including investigations concerning the structure and nature of the genus *Bodo* (Ehr.). *Proc. Boston. Soc. Nat. Hist.* **4**, 124.

CHISWELL, J. R. (1956). A taxonomic account of the last instar larvae of British Tipulidae. *Trans Roy. Ent. Soc. Lond.* **108**, 409.

CLARK, T. B. (1959). Comparative morphology of four genera of the Trypanosomatidae. *J. Protozool.* **6**, 277.

DELAMATER, E. D. (1951). A new staining and dehydration procedure for the handling of micro-organisms. *Stain. Tech.* **26**, 199.

GIBBS, A. J. (1951). *Leptomonas capsularis* n.sp. and other flagellates parasitic in *Cletus ochraceus* (Hemiptera). *Parasitology*, **41**, 128.

GIBBS, A. J. (1954). *Leptomonas cenaei* sp.nov. from the digestive tract of *Cenaeus carnifex* (Hemiptera) with special reference to metacyclic forms. *Trans. R. Soc. S. Afr.* **34**, 245.

GRASSÉ, P. P. (1952). *Traité de Zoologie*, Tome I, Fasc. I. Paris.

HINDLE, E. (1912). What is the genus *Leptomonas*? *Parasitology*, **5**, 128.

HORNE, R. W. & NEWTON, B. P. (1958). Fine structure of the kinetoplast blepharoplast complex. *Exp. Cell Res.* **15**, 103.

KENT, W. S. (1880–82). *A Manual of the Infusoria*. London.

KRAMÁŘ, J. (1950). Parasites in the larvae of the Cranefly *Tipula maxima* Poda. *Vestn. Ceskoslov. zool. Spolecn.*, **14**, 35.

LUDWIG, F. W. (1946). Studies on the protozoan fauna of the larvae of the cranefly *Tipula abdominalis*. I. Flagellates, amoebae and gregarines. *Trans. Amer. Micr. Soc.* **65**, 189.

MACKINNON, D. (1910). Herpetomonads from the alimentary tract of certain dung flies. *Parasitology*, **3**, 225.

MILNE, A., COGGINS, R. E. & LAUGHLIN, R. (1958). The determination of numbers of leatherjackets in sample turves. *J. Anim. Ecol.* **27**, 125.

MINCHIN, E. A. & THOMSON, J. D. (1915). The rat trypanosome, *Trypanosoma lewisi*, in its relation to the rat flea, *Ceratophyllus fasciatus*. *Quart. J. micr. Sci.* **60**, 463.

OLDHAM, J. N. (1928). On the final larval instar of *Tipula paludosa* Meig. and *Tipula lateralis* Meig. *Proc. R. Phys. Soc. Edin.* **21**, 217.

PROWAZEK, S. VON (1904). Die Entwicklung von *Herpetomonas*, einem mit dem Trypanosomen verwandten Flagellaten. *Arb. K. Gesundheitsamte*, **20**, 440.

SOROURI, P. (1955). The nuclear cytology of *Leishmania tropica*. *J. Morph.* **97**, 393.

WENYON, C. M. (1913). Observations on *Herpetomonas muscae-domesticae* and some allied flagellates. *Arch. Protistenk.* **31**, 1.

WENYON, C. M. (1926). *Protozoology*, I. London.

Parasitology (1960), **50**, 365–385
With 28 text-figures
Printed in Great Britain

New species and new records of Argasidae from Israel. Observations on the rudimentary scutum and the respiratory system of the larvae of the Argasidae

By O. THEODOR and M. COSTA
Department of Parasitology, Hebrew University, Jerusalem

(*Received* 31 *August* 1959)

Five species of Argasidae were recorded from Palestine in a paper on *Ornithodorus coniceps* (Theodor, 1932). Since then several additional species have been found, some of which are apparently new. Some of the identifications are tentative. Only larvae were available of some species and the larvae of many species are insufficiently known. The group of *Argas reflexus* and related species in particular is in need of a revision which is being undertaken at present by Hoogstraal.

THE RUDIMENTARY SCUTUM OF THE LARVAE OF SOME SPECIES OF ARGASIDAE

According to the generally accepted definition of the family Argasidae, a scutum is absent. There is, however, in the larvae of a number of species a plate in the middle of the dorsum closely resembling the dorsal plate of Gamasid mites which seems to represent a rudimentary scutum.

This dorsal plate is mentioned and figured by Nuttall, Warburton, Cooper & Robinson (1908) in the description of the larva of *Argas persicus* as follows: 'There is a circular or oval unwrinkled area or plate in the middle of the dorsum', and in the description of the larva of *A. vespertilionis*: 'Integument with fine transverse parallel folds except on an oval squamous shield anterior to the middle of the dorsum.' Cunliffe (1914) in his description of the larva of *A. brumpti* indicates such an elongate dorsal plate in his figure but does not mention it in the description. Neumann (1942) describes and figures the dorsal plate of the larva of *A. columbarum* (= *A. reflexus*), but considers it wrongly as part of the respiratory system (see below). Hoogstraal (1955*a*) shows this plate in the figures of *A. boueti* and *A. confusus* and in the description of the larva of *A. vespertilionis* he mentions 'an elongate, oval, squamous shield on the dorsum'.

A similar elongate, oval plate exists on the dorsum of *Argas transgariepinus* (see below, p. 377).

Cooley & Kohls (1944) mention the presence of a dorsal plate for the following American species: *Ornithodorus talaje, O. yumatensis, O. stageri, O. kelleyi* and *O. coprophilus*. They figure it for some of these species and state in the description of the larva of *O. coriaceus* that a dorsal plate is absent. In the description of the larva of *O. stageri* they state, that the dorsal plate is 'shining and with faint pits (visible with reflected light in unmounted specimens)'.

For Old World species of *Ornithodorus* the dorsal plate is mentioned by

Hoogstraal (1955*b*) in the description of the larva of *O. delanoei*. In the following the dorsal plate of a number of other Old World species is described.

In *O. lahorensis* the dorsal plate is exceptionally well developed. It is elongate, oval, 0·4 mm. long and 0·2 mm. wide and has a reticulate pattern with convex, glistening meshes. It is easily visible in fresh and in alcohol specimens. In most specimens there are two short folds in the middle (Text-fig. 1). The dorsal plate of the larva of *O. delanoei acinus* is similar but even larger (0·6 × 0·34 mm.). It is blunt posteriorly and pointed in front.

In *O. salahi* a small rounded or oval plate (0·1 mm.) is present and in *O. coniceps* and in *O. capensis* the plate is wide posteriorly and has a narrower anterior part (Text-fig. 20). In these species the plate is discernible only with difficulty, as the striations of the integument are very fine and the plate is not glistening. It is best demonstrated in specimens stained in Mayer's haematoxylin. There is no dorsal plate in the larvae of *O. savignyi*, *O. tholozani* and *O. erraticus* where striations can be clearly made out over the whole dorsal surface.

Further information on the presence or absence of the dorsal plate in larvae of Argasid ticks may assist in the grouping of the species. If the interpretation of the dorsal plate as a rudimentary scutum is accepted, the definition of the family would have to be modified to read: Scutum either absent or present in rudimentary form in the larva.

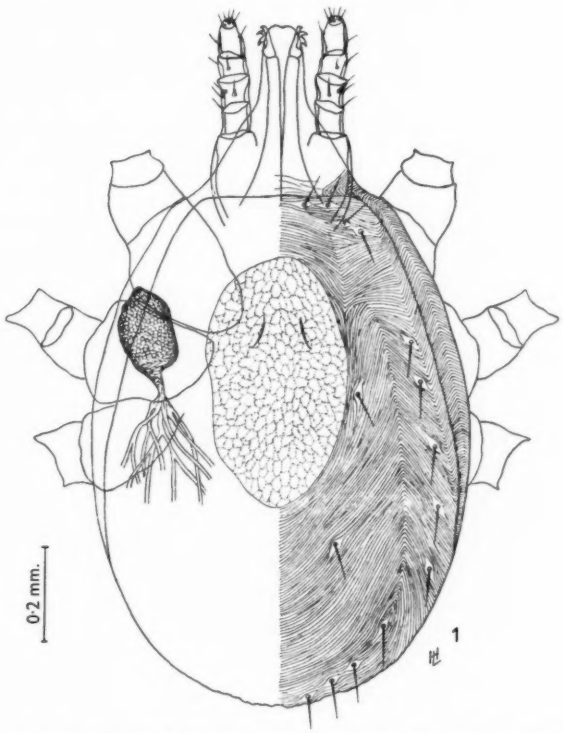
THE RESPIRATORY SYSTEM OF THE LARVA

It is generally accepted that the larvae of ticks have no respiratory system. Thus Nuttall, Cooper & Robinson (1908) in their description of the larva of *Haemaphysalis punctata* state: 'The larva possesses no respiratory (tracheal) system', and Vitzthum (1931) in Kükenthal's *Handbuch der Zoologie* says that 'Die Larven der Parasitiformes haben noch keine Atmungsorgane'.

Some authors (Delpy, 1938; Cooley, 1938) considered small oval structures posterior to the coxae of the larvae and in addition in various positions on the abdomen as spiracles without, however, demonstrating the presence of a tracheal system. The probably true nature of these structures has been recognized by Neumann (1942) and Schulze (1949) who consider them as combined dermal sense organs and glands ('Drüsensinnesorgane'). Lees (1947) considers them as dermal glands and Dinnik & Zumpt (1949) as sense organs.

Without going into the question of the function of these structures, it seems that the best indication that they are not spiracles is given by the fact that they are present in the nymph and in the adult tick, independent of, and in addition to, the fully functional respiratory apparatus. In the nymph structures of similar shape and position, but of larger size, are more numerous than in the larva, as a rule two after each coxa. In the male of some genera such structures are very numerous between the coxae and particularly anteriorly to the genital orifice. They are less numerous in the female. They are also larger and of a more complicated structure, multiple in many cases (Text-fig. 2). Thus, as far as our knowledge goes, no respiratory system has been demonstrated as yet in the larvae of the Ixodidae.

In the Argasidae, the presence of tracheae has been mentioned by Neumann (1942). He, however, wrongly considered the dorsal plate as connected with the respiratory system, acting as a kind of spiracle. He did not demonstrate the connexion of the tracheae observed by him with the dorsal plate. He gives a photograph of a section through the tracheal trunk, but did not find the spiracular opening near the coxa nor the branching of the tracheal trunk into tracheae. Schulze (1949) states that he observed in sections that a thick tracheal trunk opens into the fold between coxa 1 and 2 in *Argas columbarum* (= *A. reflexus*).

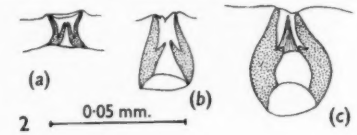


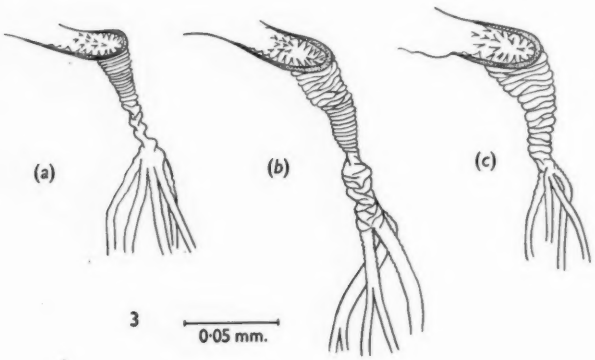
Text-fig. 1. *Ornithodorus lahorensis*, larva. Right half, dorsal view; left half, position of respiratory apparatus.

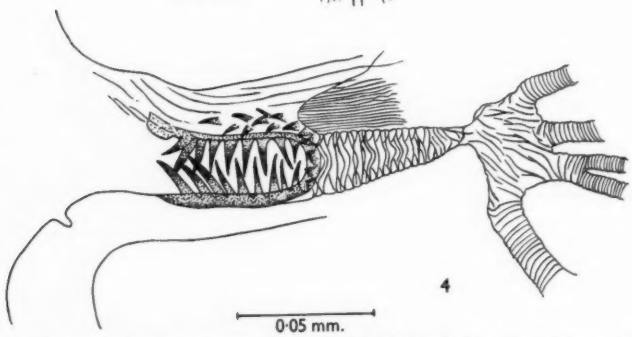
There is a well-developed respiratory system in the larvae of some species of *Argas* examined, while there is no such system in others. In *A. persicus*, *A. reflexus*, *A. hermanni* and *A. transgariepinus* there is a round or oval spiracle opening near the anterior surface of coxa 2. This opening is partly closed by spines projecting into the lumen. The spiracle leads into a tracheal trunk with more or less regular taenidia. This tracheal trunk narrows and then branches simultaneously into a number of tracheae which penetrate into the whole body cavity as in the nymphs and adults. Taenidia are visible only near the origin of the tracheae in most cases in balsam preparations (Text-fig. 3). The tracheae are best demonstrated by immersing living larvae in alcohol when the air caught inside the tracheae stands

out with its characteristic silvery sheen. In *A. transgariepinus* the spiracular opening is relatively larger and the spines inside it are longer than in the other species examined (Text-fig. 4). No respiratory system was found in larvae of *A. confusus* and *A. vespertilionis*.

In *Ornithodorus lahorensis* the atrium is particularly well developed (Text-fig. 1, 5, Pl. I, fig. 1). It is a large sac, opening in a slit between coxa 1 and 2. Its walls







Text-fig. 2. Dermal glands or sense organs of *Rhipicephalus sanguineus*: (*a*) larva, (*b*) nymph, (*c*) adult.

Text-fig. 3. Respiratory apparatus of larvae of: (*a*) *Argas* (?) *hermanni*, (*b*) *Argas* sp. from *Neophron*, (*c*) *A. persicus*.

Text-fig. 4. Respiratory apparatus of *A. transgariepinus*.

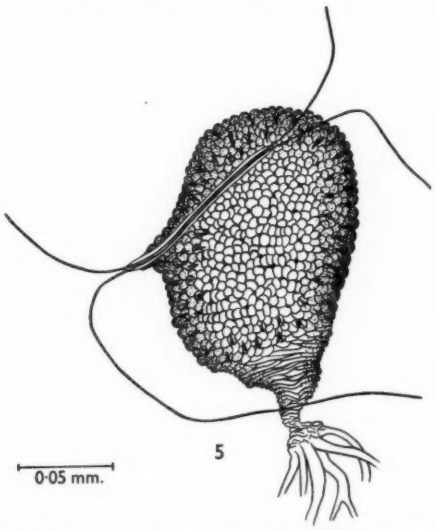
are formed by a chitinous network with spines projecting into its lumen at regular intervals. The sac narrows into a funnel-shaped tracheal trunk with a wavy taenidium and branches into a number of tracheae. Schulze (1949) observed this structure but did not find either the opening between the coxae or the tracheae. He thought that it might have some connexion with respiration because of its similarity with the peritreme of the spiracle in other species.

Larvae of *O. delanoei acinus* in the collection of the British Museum were found to resemble those of *O. lahorensis* closely. The pattern of striations on the dorsum and the dorsal plate are very similar. The respiratory system has no sac-like atrium as in *O. lahorensis*, but consists of a wide funnel with a wavy taenidium and with some spines near the opening.

The larvae of *O. lahorensis* and *O. delanoei acinus* thus differ from other species of *Ornithodorus* examined in the pattern of striations on the dorsum, the strong development of the dorsal plate and the presence of a respiratory system.

Schulze (1941) created the genus *Alveonasus* for *O. lahorensis* on the basis of differences in the structure of Haller's organ. These differences, however, seem to be of specific rank only, as he puts *O. delanoei acinus*, which is closely related to *O. lahorensis*, into the genus *Ornithodorus*, together with *O. moubata* and *O. savignyi*.

Although the reasons for the creation of the genus *Alveonasus* given by Schulze seem insufficient, the differences described above clearly distinguish *O. lahorensis*



Text-fig. 5. Respiratory apparatus of *Ornithodorus lahorensis*.

and *O. delanoei acinus* from other species of *Ornithodorus* examined. Other species of the *lahorensis* group (*O. delanoei*, *O. foleyi*) have proved to possess similar characters, and recognition of this group by name would seem to be therefore justified. The name *Alveonasus* with an emended diagnosis could be used for this group, if not as a genus, perhaps as a subgenus. Examination of the larvae of other species of *Ornithodorus* would be necessary to decide this question.

No trace of a respiratory system was found in living larvae of *O. tholozani*. They contained, however, air bubbles in their intestinal tract and it is thus possible that they breathe by swallowing air. No respiratory system was found in preserved material of *O. coniceps*, *O. erraticus* or *O. salahi*.

The position of the spiracle near coxa 2 instead of near coxa 4 as in nymphs and adults is rather surprising. It is perhaps pertinent in this connexion to mention

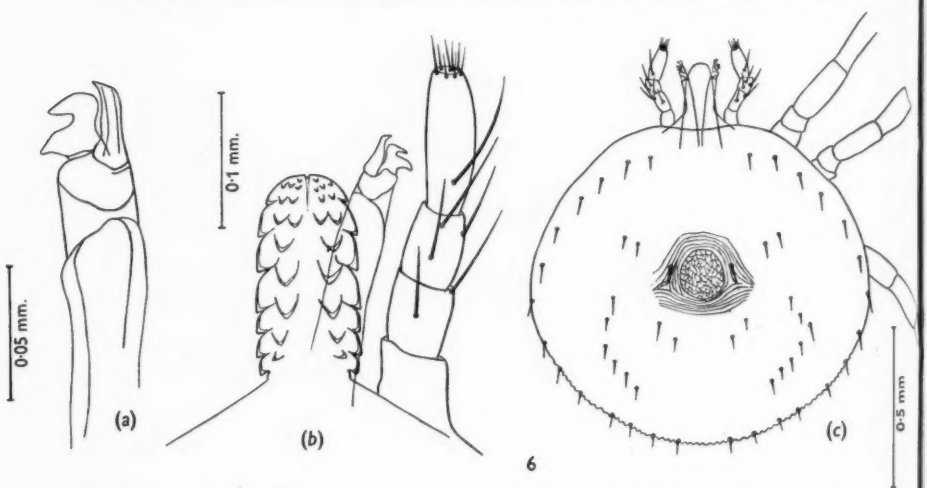
an observation by Schultze (1941) who found an adult *Aponomma* with a supernumerary spiracle of a shape found in mesostigmatic mites near coxa 1. He suggests that 'der ungewöhnliche Ort wuerde nach dem Gesetz der Isopotenz serial homologer Körperabschnitte verstaendlich sein' (Lebedinsky, 1925).

Palp formula. One of the most useful characters for the differentiation of Argasid larvae is the relative length of the palpal segments. In order to facilitate comparison of the various species, the use of a palp formula is suggested, similar to that used in the descriptions of Phlebotomus. The formula used in the following description gives the relative length of the segments in their natural arrangement, the length of segment 1 being taken as 1.

SYSTEMATIC PART

(1) *Argas persicus* (Oken) 1818

This species which was widely distributed throughout the country, mainly in the coastal plain and in the Jordan valley, has become rare in Israel since poultry breeding by modern methods and the use of modern insecticides have been intro-



Text-fig. 6. Larva of *Argas persicus*: (*a*) chelicera, (*b*) hypostome and palp, (*c*) dorsal view.

duced. Specimens have been found under the bark of a tree in the central Negev. These were either parasites of wild birds or were possibly left over from Bedouin camps which had moved in the meantime. Larvae were also found on a specimen of *Ketupa ceylonensis* (Tiberias, 17 November 1948), on *Circus* sp., June 1948 and on *Streptopelia turtur* (Beer Tuviah, 6 September 1956).

The larva has been described and figured in Nuttall, Warburton, Cooper & Robinson (1908) generally referred to as Nuttall's monograph. The palp formula is: 1–1–1–2 and the palp is 1·6 to twice as long as the hypostome which is 0·14 mm. long and 0·07 mm. wide and has two longitudinal rows of six to seven large teeth in each half. The two anterior transverse rows consist of three teeth in each half and there are three to four transverse rows of smaller teeth near the tip.

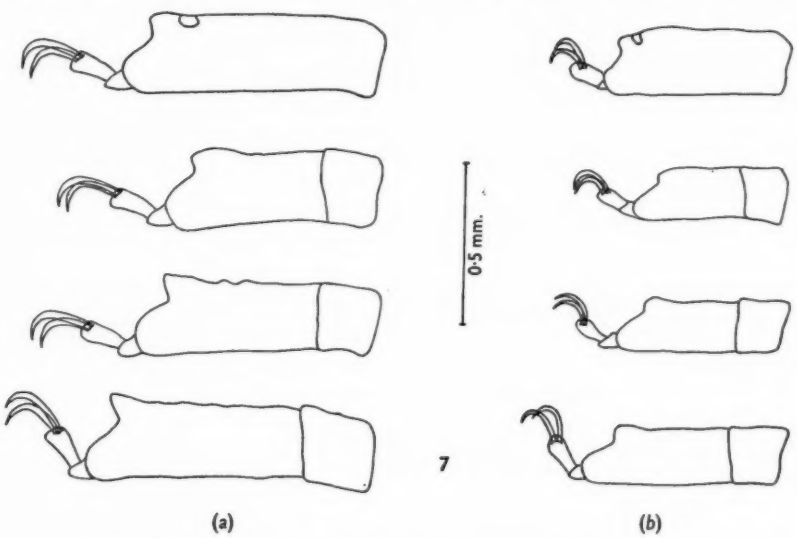
There is a rounded dorsal plate in the middle of the dorsum. The striations of the integument are rather coarse and arranged in a zig-zag pattern posteriorly. The arrangement of the hairs is shown in Text-fig. 6*c*. There is a marginal row of about twenty-six to thirty short barbed hairs, beginning near the base of the palps. Fifteen to sixteen of these hairs are in the posterior half of the body.

The spiracular opening lies at the anterior surface of coxa 2 and has short spines projecting into its lumen. Tracheal trunk with an irregular taenidium, narrowing before branching into four to five tracheae (Fig. 3*c*).

(2) *Argas reflexus* (Fabricius) 1794

(3) *Argas hermanni* Audouin, 1827

Hoogstraal (1956) mentions Theodor (1932) as having recorded *A. reflexus* from Palestine. This is not correct and the specimens recorded below are the first found in the country. Hoogstraal (1958) deletes *A. reflexus* from the list of the



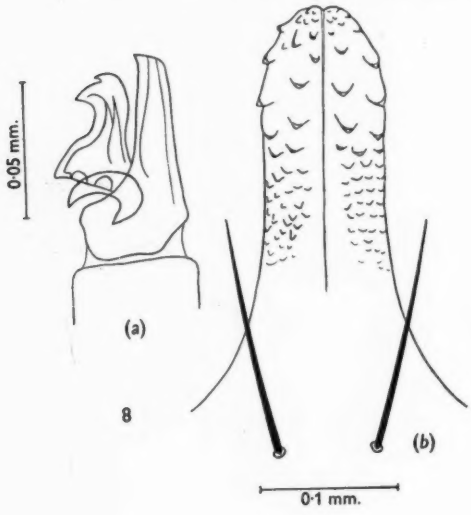
Text-fig. 7. Tarsi of legs 1–4 of: (*a*) *Argas reflexus*, (*b*) *A. hermanni*.

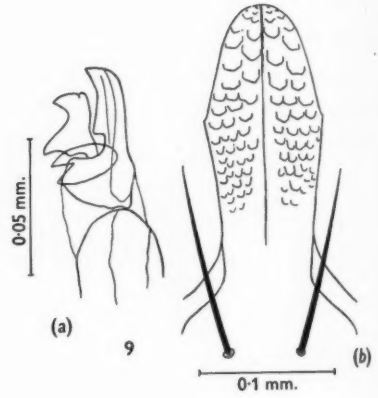
Egyptian fauna and states that *A. reflexus* is apparently restricted to Europe and that the common pigeon parasite in Egypt which was considered as *A. reflexus* is in fact *A. hermanni*.

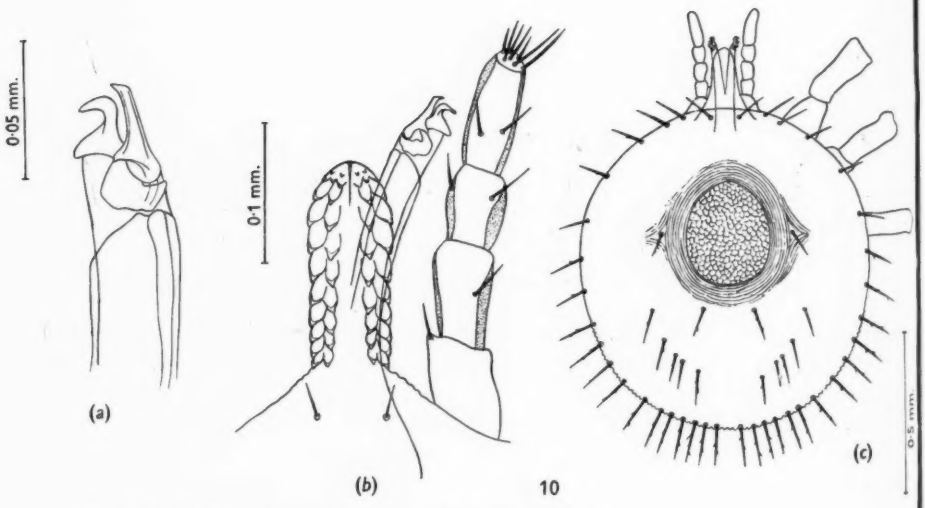
Comparison of *A. reflexus* from Germany, kindly provided by Professor H. Kemper, Berlin, with material from Egypt showed that *A. reflexus* and *A. hermanni* differ in a number of characters. *A. reflexus* is larger, the legs are thicker and the tarsal humps are more pronounced and more forwardly directed than in *A. hermanni* (Text-fig. 7). The striated margin of *A. reflexus* is wider and more clearly differentiated from the dorsal integument than in *A. hermanni*. The arrangement of the discs is different, the discs stand more closely together in *A. reflexus*,

but the appearance of the discs is rather variable and they possibly appear different according to the state of gorging in which the nymph moults.

The hypostome of *A. reflexus* is parallel-sided and has two to three transverse rows of large teeth anteriorly and several rows of much smaller teeth near the tip. Behind the large teeth there are several irregular rows of small teeth (Text-fig. 8*b*). In *A. hermanni* the hypostome tapers markedly anteriorly, the teeth are scale-like







Text-fig. 8. Hypostome and chelicera of *Argas reflexus*.

Text-fig. 9. Hypostome and chelicera of *A. hermanni*.

Text-fig. 10. Larva of *Argas* (?) *hermanni*: (*a*) chelicera, (*b*) hypostome and palp, (*c*) dorsal view.

and decrease gradually in size towards the base (Text-fig. 9*b*). A good illustration of it is given in Nuttall's monograph (vol. 1, p. 28, fig. 35, after Neumann).

The distal hook of the movable process of the chelicerae has a well-developed secondary tooth in *A. reflexus* which is practically absent in *A. hermanni* (Text-figs. 8*a*, 9*a*).

The palp formula is: 1–1·25–0·65–0·75 in *A. reflexus* and 1–1–0·7–0·7 in *A. hermanni*, that is, segment 2 is relatively longer in *A. reflexus*.

In Israel both *A. reflexus* and *A. hermanni* occur. A single female of *A. reflexus* was found in Jerusalem near a pigeon cote on 18 January 1954. A female of *A. hermanni* was found in Jerusalem on 2 May 1951. The host of this latter specimen is not known. A large nymph belonging probably to *A. reflexus* was found under a stone in Wadi Ramon in the central Negev.

The larva of *A. reflexus* has not been described in detail to our knowledge. Brumpt (1949) gives a figure which, however, lacks essential details and Hoogstraal (1952) mentions in a key that the larva differs from that of *A. persicus* in having the palps 1·1 to 1·4 times as long as the hypostome and having about forty hairs at the posterior border of the abdomen.

Larvae resembling those of *A. reflexus* were found on a number of wild birds, mainly on *Corvus rhipidurus*. These larvae differ from larvae of *A. reflexus* in a number of characters and they are possibly the larvae of *A. hermanni*. They may, however, prove to belong to another species. The larva of *A. hermanni* has not been described.

The following is the description of larvae found repeatedly on *Corvus rhipidurus* and more rarely on *Coracias garrulus* (Text-fig. 10).

Length of body without hypostome 0·6 mm.

Palp formula: 1–1·3–1–1·5. Hypostome 0·15 mm. long, 0·06 mm. wide, with two longitudinal rows of eight to nine teeth in each half. Two to three rows of smaller teeth near the tip. Dorsal plate large, broadly oval, 0·3 × 0·24 mm. with reticulate surface. Marginal row of hairs complete. Hairs barbed, longer than in *A. persicus*. There are thirty-six hairs in the row, about twenty-eight of which are in the posterior half of the body, where they stand more closely together than in the anterior half (Text-fig. 10*c*). The distal hook of the movable process of the chelicerae is slender without secondary tooth. The spiracular opening is at the anterior surface of coxa 2 and has some short spines projecting into the lumen. The taenidium of the tracheal trunk is very regular (Text-fig. 3*a*)*.

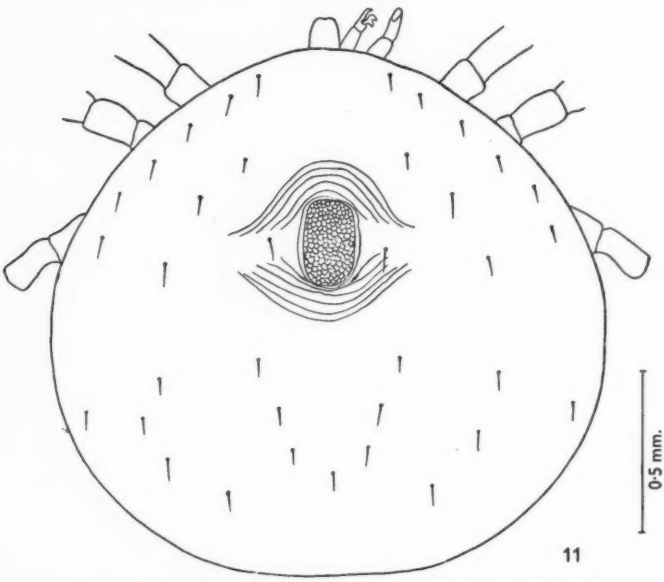
* After the paper went to press the larvae from *Corvus rhipidurus* were compared with larvae of *A. hermanni* from Egypt. They differ from them mainly in the form of the distal hook of the movable process of the chelicerae which is broader and has a small secondary tooth in *A. hermanni* and in the arrangement of the teeth of the hypostome which has 2 transverse rows of 3 teeth on each side near the tip in *A. hermanni*. The palp formula of *A. hermanni* is 1–1·5–1–1·8.

From the larvae of *A. reflexus* the larvae from *Corvus rhipidurus* also differ in the form of the distal hook of the movable process of the chelicerae which has a well developed secondary tooth in *A. reflexus* and in the number of hairs of the marginal row of the abdomen which is over 50 in *A. reflexus*.

(4) *Argas* sp.

A number of larvae were found on a specimen of *Neophron percnopterus* in Lahavoth Habashan north of Lake Huleh, 25 May 1959 which resemble those of *A. persicus* superficially, but differ in a number of characters. *Neophron* is widely distributed in the Mediterranean, eastward to Belujistan, and winters in Africa south of the Sahara. Although the larvae probably belong to a new species, it does not seem advisable to name them before more information about its later stages and their distribution is available.

Description. Palp formula: 1–1·3–1–2. Segment 2 relatively longer than in *A. persicus*. Hypostome 0·2 mm. long, 0·1 mm. wide. Only two longitudinal rows of teeth in each half, reaching to the tip. There are eight large teeth in each row

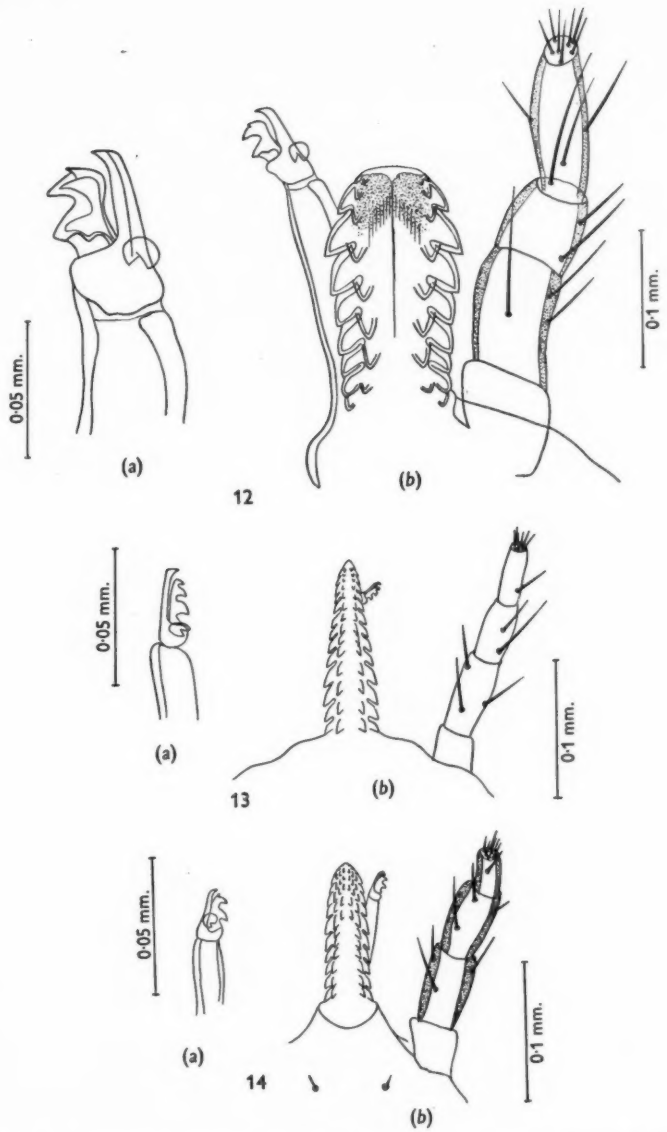


Text-fig. 11. Larva of *Argas* sp. from *Neophron percnopterus*.

and one or two smaller ones anteriorly. There are no teeth in a wide median stripe up to the anterior margin (Text-figs. 11, 12).

The dorsal plate is small, oval, 0·25 × 0·15 mm. Marginal row of hairs on the dorsum incomplete. There are about six widely spaced short hairs at the anterior margin on each side and none at all at the margin of the posterior half. There are two hairs at the sides of the dorsal plate and about sixteen hairs in several longitudinal rows in the posterior half of the dorsum.

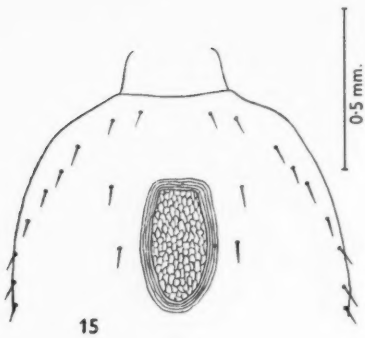
Respiratory system as in *A. persicus* with an irregular taenidium near the opening which becomes more regular towards the branching point of the tracheae (Text-fig. 3*b*).

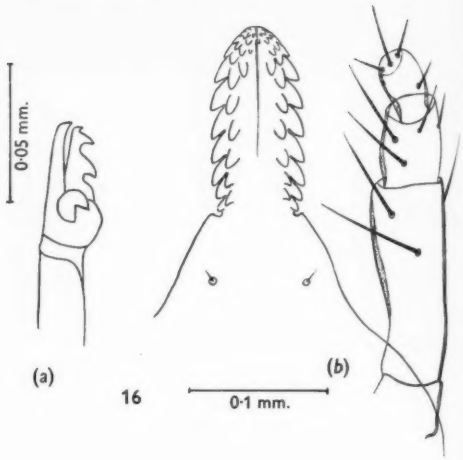


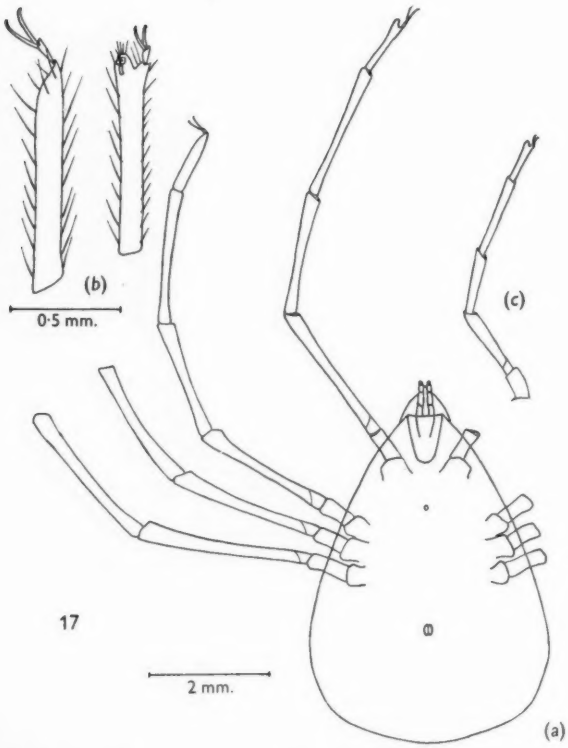
Text-fig. 12. Larva of *Argas* sp. from *Neophron percnopterus*: (*a*) chelicera, (*b*) hypostome and palp.

Text-fig. 13. Larva of *Argas confusus*: (*a*) chelicera, (*b*) hypostome and palp.

Text-fig. 14. Larva of *A. vespertilionis* from Sicily: (*a*) chelicera, (*b*) hypostome and palp.







Text-fig. 15. Larva of *Argas transgariepinus*. Dorsal plate.

Text-fig. 16. Larva of *A. transgariepinus*: (*a*) chelicera, (*b*) hypostome and palp.

Text-fig. 17. *Argas* sp. near *A. boueti*. Exuvia of large nymph: (*a*) ventral, (*b*) tarsus 1 and 2, (*c*) foreleg of *A. boueti* for comparison.

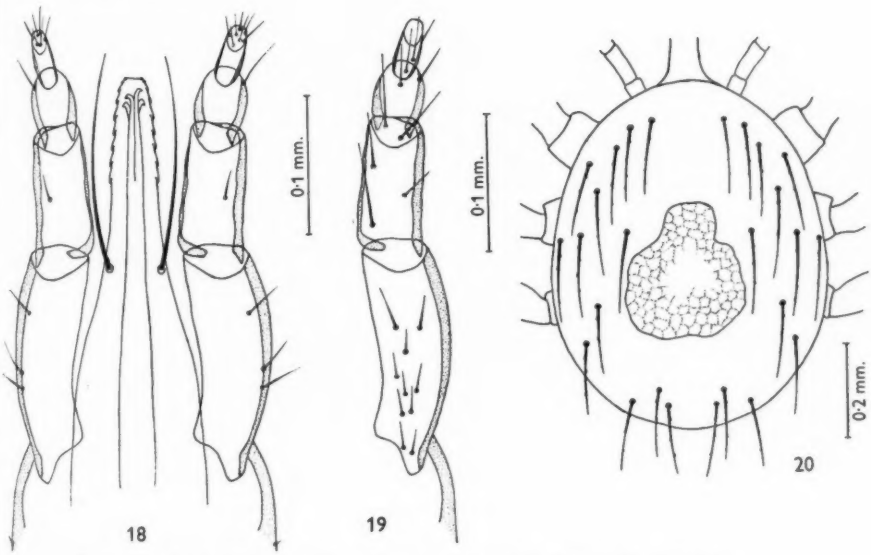
(5) *Argas confusus* Hoogstraal, 1955

A. confusus was separated recently from *A. vespertilionis*. *A. confusus* is restricted to Africa as far as previous records indicate, while *A. vespertilionis* is cosmopolitan. The larvae of both species are very similar and, according to Hoogstraal, the larva of *A. confusus* resembles that of *A. boueti* closely.

A few larvae found are provisionally attributed to *A. confusus*. They differ from the larva of *A. vespertilionis* mainly in the arrangement of the teeth on the hypostome which has only two files as against several near the tip in *A. vespertilionis* (Text-figs. 13, 14). Palp formula: 1–2·1–1·7–1·8. No respiratory system was found in these larvae or in larvae of *A. vespertilionis* from Sicily.

Two larvae from *Rhinolophus ferrum equinum*, Jerusalem, 6 April 1937.

Six larvae from *Taphozous nudiventris*, Wadi Amud, near Lake Tiberias, 29 December 1940.



Text-fig. 18. *Argas* sp. near *A. boueti*. Capitulum of nymph, ventral.
Text-fig. 19. *Argas* sp. near *A. boueti*. Palp of nymph, dorsal.
Text-fig. 20. *Ornithodorus coniceps*. Dorsal view of larva with dorsal plate.

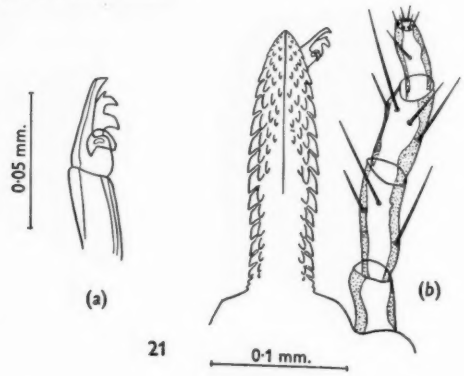
(6) *Argas transgariepinus* White, 1846

The species has been redescribed by Hoogstraal (1957). Only a single larva was found on *Plecotus auritus christiei* in Eilath (Akaba) on 3 March 1957. The following details are added.

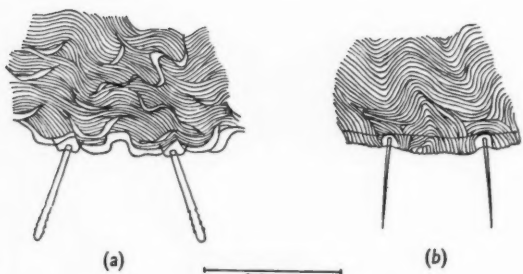
Palp formula: 1–4·5–2–1·6. Dorsal plate well marked, oval, 0·35 × 0·18 mm. It has a reticulate pattern, each mesh convex and glistening (Text-figs. 15, 16). Respiratory system (Text-fig. 4). Spiracular opening relatively large, oval, at the anterior surface of coxa 2. There are numerous long spines projecting into its lumen. Tracheal trunk short, with clearly marked taenidium, tapering before branching into six to eight tracheae.

(7) *Argas* sp. near *Argas boueti* Roubaud & Colas-Belcour, 1933

In 1934 two exuviae were found in the large cave at Sdom (Djebel Usdum) at the south end of the Dead Sea which belong to a species related to *A. boueti*. In spite of repeated visits to the cave no specimens of the tick itself could be found. Although this tick will probably prove to belong to a new species, it seems not advisable to name it from an exuvia. A provisional description is given as the chances of finding the tick are remote. This is the tick referred to by Hoogstraal (1954, 1955, 1956) as 'a long-legged variety of *A. boueti*'. Hoogstraal gives the locality erroneously as Jerusalem.



21



22

Text-fig. 21. *Ornithodorus coniceps*, larva: (*a*) chelicera, (*b*) hypostome and palp.
Text-figure 22. Structure of integument and hairs at posterior margin of body of: (*a*) *O. erraticus*, (*b*) *O. tholozani*.

Description (Text-figs. 17–19). Exuvia of large nymph, probably the last stage before moulting into adult. Length of body 6 mm. Greatest width posteriorly 4 mm. Body pear shaped. Integument finely mammillated. No marginal suture. Hood projecting beyond anterior margin. Capitulum long and cylindrical. Palps tapering. Formula: 1–0·7–0·3–3·0. Hypostome 3·5 times as long as wide, with a single row of six to seven weak denticles laterally in the distal half of the hypostome. Two very long hypostomal hairs which reach beyond the tip of the hypostome. Anus broadly elliptical at the posterior two-thirds of the body with three minute hairs in each half.

Legs very long. Foreleg 9 mm, second leg 8 mm. long. Legs 3 and 4 broken beyond tibia, but femur 4 even longer than femur 1. Tarsus 1 with distal hump. Tarsus 2 tapering at tip with claws which are twice as long as those of tarsus 1. (The foreleg of a female of *A. boueti* of 5 mm. body length is 4·25 mm. long.)

Second exuvia of a younger nymph, damaged, resembling the described exuvia.

(8) *Ornithodorus coniceps* (Canestrini) 1890

This species has been fully described (Theodor, 1932). The paper was based on material from Kalendieh, near Jerusalem. The species is restricted mainly to hilly country and needs high humidity, particularly for the eggs and early stages.

The hypostome of the larva is characteristic for the group of species including *O. capensis*, *O. talaje*, *O. kelleyi* and *O. dunni*. The only details to be added to the description are the presence of a dorsal plate which is wide posteriorly and narrower anteriorly (Text-figs. 20, 21). The palp formula is: 1–1·5–1·5–1. Palps very slender. Post-hypostomal hairs minute. Respiratory system absent. Dorsal process of chelicerae of adult tick with three teeth.

New records:

Rama, Upper Galilee, in chicken house, 20 August 1956.

Rosh Pinah, Upper Galilee, in chicken house, 14 July 1952.

Ras en Nakura, in nest of *Sterna hirundo*, 15 July 1955.

Kfar Masaryk, near Acre, a larva on *S. hirundo*, 18 June 1959.

Sussita, near Lake Tiberias, in nest of *Petronia petronia* and in nest of *Columbia livia*, 6 June 1957.

Kabara, cave in foot hills of Mt. Carmel, near nest of *C. livia*, 29 August 1954.

Some of the above records are the first from localities in the plain and indicate that the high humidity in the nest of cave breeding birds or in the nest of water birds are the main factors determining distribution and not the general climatic conditions.

(9) *Ornithodorus tholozani* (Laboulbène & Megnin) 1882 (Pl. I, fig. 2)

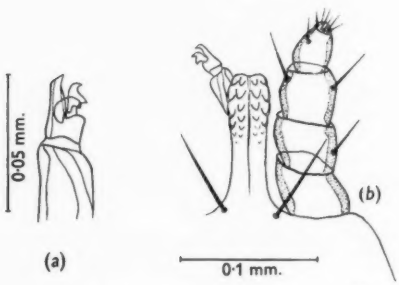
This species is widely distributed throughout the country, in the hills and in the plains, from Upper Galilee in the north to near Beersheba in the south. It is generally found in caves, where it hides in the ground, sometimes to a depth of 20 cm. It feeds on rodents and is often found in their burrows. It was also found near bat colonies in a cave near Herzliah and has been found feeding on *Rhinopoma microphyllum* in Tiberias (30 January 1952). It was found in the ruins of the crusader's castle of Kala'at Jeddin (Jechiam) in Upper Galilee where it caused an outbreak of relapsing fever in July 1947 and in an old house in Mishmar Hayarden south of Lake Huleh in October 1946. Its most southern locality found is a cave in Darajat, near Tel Arad, about 30 km. east of Beersheba.

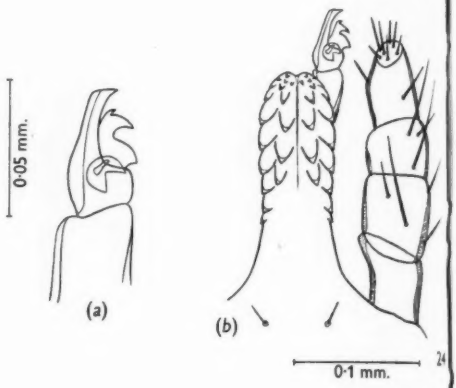
The larva has apparently not been described.

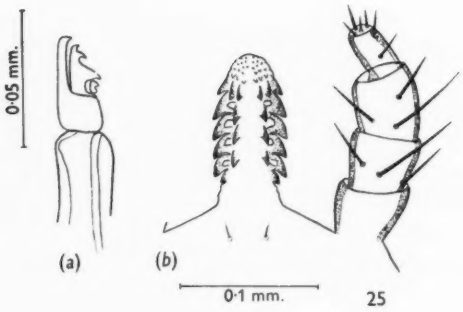
Length of unfed larva 0·75 mm., width 0·6 mm. Integument finely striated, in a zig-zag pattern posteriorly (Fig. 22*b*). There is no dorsal plate. There is a complete marginal row of about twelve to fourteen long hairs anteriorly and eight to ten in the posterior half of the body. Two such hairs in the middle of the anterior

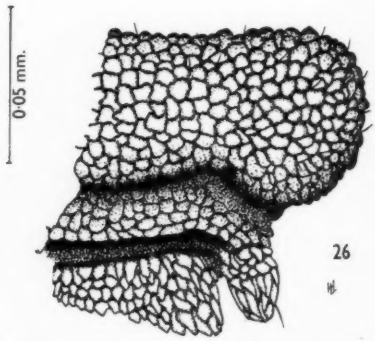
half of the dorsal surface and 4 in the posterior half. The hairs of the ventral surface are shorter and thinner.

Hypostome 0·08 mm. long and 0·035 mm. wide, narrower basally and with 2/2 files of coarse teeth in the anterior half, about four in each file (Text-fig. 23*b*). A few weak teeth further basally. Two long post-hypostomal hairs which reach nearly to the tip of the hypostome. Palps short and thick, formula: 1–0·7–0·7–0·7 (Text-fig. 23*b*). Respiratory system absent. (Dorsal process of chelicerae of adult with four to six teeth (Text-fig. 28*f*).









Text-fig. 23. Larva of *Ornithodorus tholozani*: (*a*) chelicera, (*b*) hypostome and palp.
Text-fig. 24. Larva of *O. lahorensis*: (*a*) chelicera, (*b*) hypostome and palp.
Text-fig. 25. Larva of *O. salahi*: (*a*) chelicera, (*b*) hypostome and palp.
Text-fig. 26. *O. procaviae* n.sp. Anterior part of female, profile.

The larva of *Ornithodorus erraticus* from Algeria is rather similar. The hairs on the dorsal surface are clubbed and the integument is mammillated in the posterior part of the body (Text-fig. 22*a*).

(10) *Ornithodorus lahorensis* (Neumann) 1908

This is a parasite of sheep and goats and has been found mainly in hilly country, in Jerusalem, Nablus, Rosh Pinah. It was found to be numerous in a cave in

Darajat, near Tel Arad where sheep were kept, near the cave mentioned above for *O. tholozani*.

The larva has apparently not been described. Brumpt (1936) gives a figure of the larva which, however, does not give essential details. The larva possesses a number of unusual characters as pointed out above in the discussion of the dorsal plate and the respiratory system.

Length of body of unfed larva 0·9 mm., width 0·6 mm. Integument finely striated, forming a pattern as shown in Text-fig. 1. A large dorsal plate in the middle of the dorsum (0·4 × 0·2 mm.). Two hairs near the sides of the plate and two or four further posteriorly. There is a complete marginal row of about twenty-five short hairs (Text-fig. 1).

Capitulum projecting anteriorly, cone-shaped. Hypostome 0·12 mm. long, 0·06 mm. wide, with 2/2 files of teeth in its anterior half, five to six teeth in each row. A few small teeth anteriorly. Chelicerae with strong hooks, the distal hook of the movable process with a secondary tooth. Dorsal process with 2 teeth. Palps short and thick, formula: 1–1·25–0·9–1·25 (Text-fig. 24).

Respiratory system exceptionally well developed. The spiracle opens in a long slit between coxa 1 and 2 and has a wide atrium with reticulate walls and spines projecting into its lumen. It narrows into a tracheal trunk which branches into a number of tracheae (Text-fig. 5 and Pl. I, fig. 1).

(11) *Ornithodorus salahi* (Hoogstraal) 1953

This species was found on *Rousettus aegyptiacus* in a cave in Herzliah together with specimens of *Ornithodorus tholozani* in 1951. It was described from Cairo, also from *Rousettus aegyptiacus*. It has not been found elsewhere in the meantime and is apparently a specific parasite of *Rousettus*. Hoogstraal, in a footnote in his paper on *O. salahi* mentions that it has been found in Jerusalem. This is not correct, the only locality in which it has been found so far being Herzliah in the coastal plain. This mistake has been taken over by Leeson (1955) in his second paper on the distribution of species of *Ornithodorus*.

The larva has been described by Hoogstraal and the only details to be added are that the larva has a small dorsal plate (0·1 mm.). Palp formula: 1–1·5–1·5–1. The three basal segments are thick and the fourth segment is much thinner (Text-fig. 25).

(12) *Ornithodorus savignyi* (Audouin) 1827

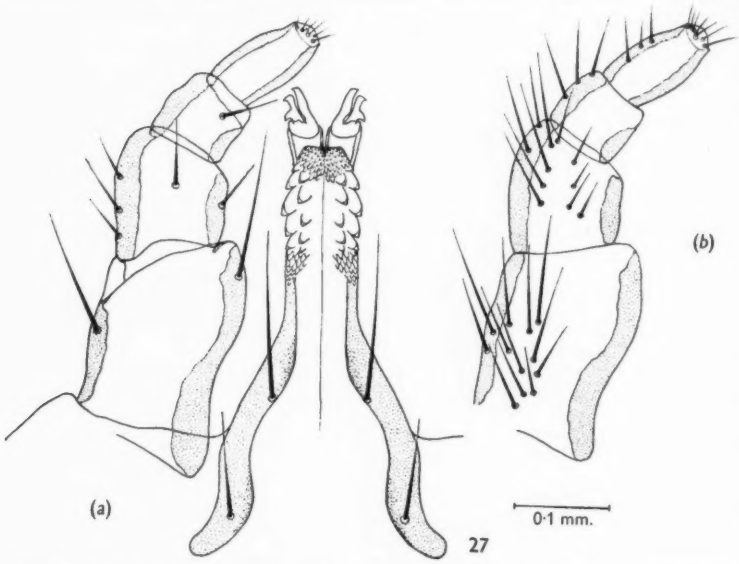
This species has not been found again since two specimens were found in the Negev in 1929. Theiler & Hoogstraal (1955) record it from El Arish and various localities in Giza province in Egypt.

(13) *Ornithodorus procaviae* n.sp.

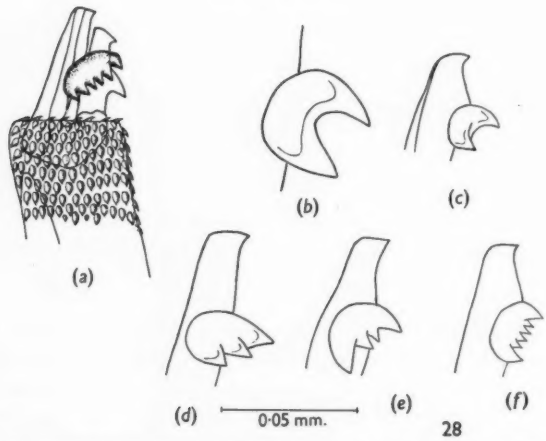
Several nymphs of this species were found on *Procavia capensis syriaca* in Lahavoth Habashan, north of Lake Huleh in 1957 and 1958. These nymphs were fed on white rats and two males and two females were obtained. No eggs have been laid so far. It proved impossible to find the ticks in the hyrax burrows which are deep in rock clefts or under a layer of several metres of large blocks of basalt.

DESCRIPTION

Female, length 5·5 mm., width 4·5 mm. Male, length 4·5 mm., width 3·5 mm. Colour brownish, legs yellowish. Shape regularly oval, rounded anteriorly, slightly wider posteriorly. Capitulum not visible dorsally. Outer rim raised in a thick ridge in unfed specimens. Discs on the dorsal surface very marked, their arrangement as in Pl. I, fig. 3. Three pairs in the middle anteriorly, forming a hexagon. A longitudinal median furrow posteriorly and a row of discs around the periphery.



Text-fig. 27. *Ornithodorus procaviae* n.sp.: (*a*) hypostome and palp, ventral; (*b*) palp, dorsal.



Text-fig. 28. *O. procaviae* n.sp.: (*a*) chelicera and dorsal process,. Dorsal process of: (*b*) *O. lahorensis*, (*c*) *O. erraticus*, (*d*) *O. coniceps*, (*e*) *O. megnini*, (*f*) *O. tholozani*.

Integument finely mammillated. Dorso-ventral grooves clearly visible as incisions of the lateral margin (Text-fig. 26, Pl. I, figs. 3, 4).

Capitulum covered anteriorly by a narrow curved hood which bears two slightly barbed setae at the dorsal surface. There are two large triangular cheeks which are heavily ridged and incised at the ventral margin. Palp formula: 1–0·6–0·36–0·5. Chaetotaxy of palp as in Text-fig. 27. Hypostome 0·17 mm. long, 0·07 mm. wide. It bears 2/2 files of large teeth, four to five in each longitudinal row. There are about five rows of small teeth near the tip and several rows of small teeth proximal to the large teeth, mainly at the sides of the hypostome. There are two long post-hypostomal setae reaching to the middle of the second palp segment in the type female and nearly to the end of the palp in the male allotype. Post-palpal hairs much shorter and more widely spaced. The membrane of the cheliceral shaft with about ten rows of large scales ventrally near the end. These scales do not become gradually smaller posteriorly as in some other species, but stop abruptly. Dorsal process of the chelicerae with about six equal teeth (Text-fig. 28*a*).

Genital aperture of the female broad, at the level of the anterior margin of coxa 2, with a slit slightly concave anteriorly. Genital opening of the male between coxae 1, half circular, with some circular folds of integument posterior to it. The pre-anal groove forms a rounded arc anteriorly and turns obliquely laterally, reaching the sides. Anus elliptical, at half the distance between the genital aperture and the posterior margin of the body. The post-anal median groove is broad anteriorly, becomes narrow where it crosses the transverse post-anal groove and widens again near the posterior margin. The transverse post-anal groove has finely striated anterior and posterior lips. Both its halves meet at the median groove at an obtuse angle. Each half is shallowly sinuate. They do not reach the sides. Coxal and supra-coxal folds well marked. Eyes absent, spiracles lateral to coxae 4.

Legs short, tarsi with some shallow, indistinct humps on leg 1, tarsi of leg 2–4 practically straight. Coxae decreasing in size posteriorly.

Larva unknown.

This species differs markedly from the other Old World species of the genus. It resembles *O. moubata* and *O. savignyi* in its rounded anterior margin and the ventral position of the hood. The scaling of the cheliceral membrane is distinct. The dorsal process of the digit of the chelicerae has six equal teeth. This character has not been used systematically as far as we are aware and figures of the dorsal process of several other species of *Ornithodorus* available are therefore given (Text-fig. 28). *O. lahorensis* and *O. erraticus* have only two teeth on the process, *O. coniceps* has three, *O. megnini* has four teeth of which the two outer ones are much larger. *O. tholozani* has four to six teeth of which the two outer ones are larger.

In addition to the morphological differences, the fact that this species is apparently a specific parasite of *Procavia* belonging to an order of such isolated systematic position, justifies its separation as a new species.

Female holotype and male allotype, one female and one male paratype in the collection of the Department of Parasitology, Hebrew University.

SUMMARY

1. The dorsal plate which probably represents a rudimentary scutum in some Argasid larvae is discussed and described.

2. The respiratory system of the larvae of some species of *Argas* and *Ornithodorus* is described.

3. The systematic position of the *lahorensis* group of the genus *Ornithodorus* is discussed and the use of the name *Alveonasus* with an emended diagnosis for this group is suggested.

4. *Ornithodorus procaviae* n.sp. is described. Two other probably new species are described provisionally, but not named.

5. New records for other species of Argasidae in Israel are given.

REFERENCES

BRUMPT, E. (1936). Evolution expérimentale de *Ornithodorus lahorensis*. Similitude biologique des estades post-embryonnaires de ce argasiné et de ceux de l'*O. megnini*. Rôle pathogène eventuel. *Ann. Parasit. hum. comp.* **14**, 632.

BRUMPT, E. (1949). *Précis de Parasitologie* (6 ed.) Paris: Masson et Cie.

COOLEY, R. A. (1938). The genera *Dermacentor* and *Otocentor* in the United States, with studies on variation. *Nat. Inst. Hlth. Bull.* no. 171.

COOLEY, R. A. & KOHLS, G. M. (1944). The Argasidae of North America, Central America and Cuba. *Amer. Midl. Nat.* monogr. no. 1.

CUNLIFFE, N. (1914). Observations on *Argas brumpti* Neumann, 1907. *Parasitology*, **6**, 379.

DELPY, L. (1938). Morphologie et disposition des stigmates respiratoires chez les larves hexapodes des Ixodides. *Bull. Soc. Path. exot.* **31**, 298.

DINNIK, J. & ZUMPT, F. (1949). The integumentary sense organs of the larvae of the Rhipicephalinae. *Psyche*, **56**, 1.

HOOGSTRAAL, H. (1952). Notes on Egyptian ticks, I. The genus *Argas* in the Cairo area. *Proc. Egypt. Ass. Sci.* **7**, 114.

HOOGSTRAAL, H. (1954). Ticks and their medical relationships in the Near East. *J. Egypt. publ. Hlth. Ass.* **29**, 1.

HOOGSTRAAL, H. (1955*a*). Bat ticks of the genus *Argas*. I. The subgenus *Chiropterargas*. *Fieldiana Zool.* **37**, 579.

HOOGSTRAAL, H. (1955*b*). *Ornithodorus delanoei* Roub. & Colas Belcour, 1931, its identification and distribution, incidence and habitat in Egypt. *Bull. Soc. Path. exot.* **48**, 734.

HOOGSTRAAL, H. (1956). African Ixodoidea. I. Ticks of the Sudan. *Research report, U.S. Naval Med. Res. Unit.* no. 3.

HOOGSTRAAL, H. (1957). Bat ticks of the genus *Argas*. 2. *Secretargas* new subgenus and *A. transgariepinus* White, 1846, its adult and immature stages; with a definition of the subgenus *Argas*. *Ann. ent. Soc. Amer.* **50**, 544.

HOOGSTRAAL, H. (1958). Bat ticks of the genus *Argas*. 3. The subgenus *Carios*, a re-description of *Argas vespertilionis* (Latreille, 1802) and variation within an Egyptian population. *Ann. ent. Soc. Amer.*, **51**, 19.

HOOGSTRAAL, H. & KAISER, M. N. (1958). The ticks of Egypt. A brief review and keys. *J. Egypt. publ. Hlth. Ass.* **33**, 51.

LEBEDINSKY, N. G. (1925). Die Isopotenz allgemein homologer Koerperteile des Metazoenorganismus. *Abh. theoret. Biol.* **22**.

LEES, A. D. (1947). Transpiration and the structure of the epicuticle in ticks. *J. exp. Biol.* **23**, 379.

LEESON, H. S. (1955). Further notes on the geographical distribution of Old World species of *Ornithodorus*. *Bull. ent. Res.* **46**, 747.

NEUMANN, K. W. (1942). Die Dorsalplatten der Argasiden als Teil eines Atmungsorgans und die angeblichen Atemplatten der Ixodiden. *Z. Morph. Ökol. Tiere*, **38**, 420.

NUTTALL, G. H. F., WARBURTON, C., COOPER, W. F. & ROBINSON, L. E. (1908). *Ticks, a monograph of the Ixodoidea.* I. *Argasidae.* Cambridge University Press.

NUTTALL, G. H. F., COOPER, W. F. & ROBINSON, L. E. (1908). The structure and biology of *Haemaphysalis punctata* Can. & Fanz. *Parasitology*, **1**, 152.

SCHULZE, P. (1941). Eine Zecke mit ueberzaehligen Atemplatten vom Muster einer anderen Milbengruppe, der Mesostigmata. *Zool. Anz.* **135**, 252.

SCHULZE, P. (1941*b*). Das Geruchsorgan der Zecken. *Z. Morph. Ökol. Tiere*, **37**, 491.

SCHULZE, P. (1942). Ueber Hautsinnesorgane der Zecken, besonders über eine bisher unbekannte Art von Arthropodensinnesorganen, die Krobylophoren. *Z. Morph. Ökol. Tiere*, **38**, 379.

SCHULZE, P. (1949). Die wahre Natur der sog. Larvenstigmen bei den Schildzecken. *Biol. Zbl.* **68**, 488.

THEILER, G. & HOOGSTRAAL, H. (1955). The identity of *Ornithodorus savignyi* and *O. pavimentosus.* Neumann, 1901. *J. Parasit.* **41**, 245.

THEODOR, O. (1932). Ueber *Ornithodorus coniceps* Can. in Palaestina. *Z. Parasitenk.* **5**, 69.

VITZTHUM, H. (1931). In Kükenthal, *Handbuch der Zoologie*, **3**, 2 (3), 36. Berlin & Leipzig: W. de Gruyter.

EXPLANATION OF PLATE 1

Fig. 1. Atrium of the respiratory system of the larva of *Ornithodorus lahorensis*.

Fig. 2. *O. tholozani*. ♀, *dorsal*.

Fig. 3. *O. procaviae* n.sp. ♀, dorsal.

Fig. 4. *O. procaviae* n.sp. ♀, ventral.

Fig. 1

Fig. 2

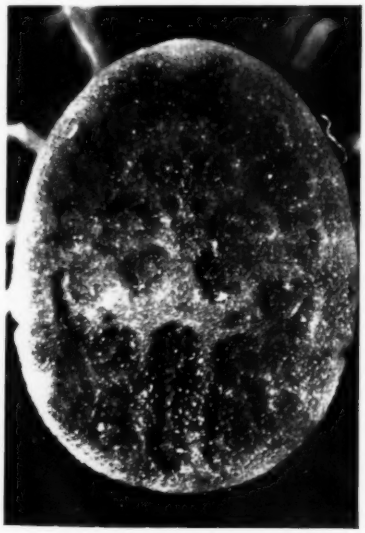


Fig. 3

Fig. 4

Parasitology (1960), **50**, 387–399
With 3 text-figures
Printed in Great Britain

Spring rise in faecal worm-egg counts of housed sheep, and its importance in nutritional experiments

By A. C. FIELD, M. R. BRAMBELL and J. ALLAN CAMPBELL
Biochemistry and Parasitology Departments, Moredun Institute, Gilmerton, Edinburgh, 9

(*Received* 19 *October* 1959)

INTRODUCTION

While investigating the effects of dietary changes on the levels of certain blood constituents of lactating ewes, a marked fall in the packed red-cell volume (P.C.V.) was observed to follow an abrupt change from hay to spring rye-grass. Towards the end of this work the animals were found to have very high faecal worm-egg counts although they had been kept indoors for some weeks. Earlier records of the worm-egg output had, unfortunately, not been taken. The fall in P.C.V. could *a priori* have been caused by the change from hay to rye-grass, or by the nematode worm burden, or by haemodilution (e.g. a post haemorrhagic anaemia associated with the loss of blood due to sampling).

To examine these possibilities further, the first of the present experiments was planned, in which animals were housed under conditions designed to prevent reinfestation by strongyloid worms, some of the animals being fed cut rye-grass over part of the experimental period (Expt. 1). A second experiment under similar conditions of management was carried out with another group of animals to examine the joint effect of pregnancy, parturition, and lactation on worm-egg output (Expt. 2).

MATERIALS AND METHODS

Expt. 1 was started on 5 February 1959, when twenty-one pregnant 3-year-old Greyface ewes were transferred from the field to three adjoining concrete pens in a fully enclosed sheep house. They were fed a daily diet of $\frac{1}{2}$ lb. ewe pencils (British Oil and Cake Mills), and $\frac{1}{4}$ lb. dried sugar beet pulp each, together with unlimited hay. The lambing period extended from 15 March to 2 April with a mode around 24 March. The second lamb from each of sixteen twin births was removed within 48 hr.

To prevent reinfestation with gastro-intestinal nematodes the following procedure was adopted: the pens, covered with wheat straw, were cleaned out daily. Once a week the floors and walls were treated with a strong caustic soda solution and after 1–2 hr. thoroughly washed with water. The pens were treated separately and during their cleaning the animals were kept in the access passage which had been treated previously with caustic soda. Access to the sheep house was through a caustic soda foot bath to prevent the introduction of infective nematode larvae on boots and implements.

Examination of lamb faeces by a zinc-sulphate flotation technique revealed no strongyloid eggs up to 12 June, when the experiment was terminated, and it is concluded that the lambs had remained uninfested by strongyloid worms. This justifies the assumption that reinfestation of the ewes by these worms was successfully prevented by the system of management. Of worms other than members of the Strongyloidea, only *Strongyloides papillosus* were identified, and for their control the procedure adopted was inadequate, since some lambs became infested with them. Reinfestation of the ewes by *S. papillosus* was not considered to have been important, however, since the infestation was patent in only five individuals, and their egg counts never exceeded 200 eggs/g. The results are only concerned, therefore with infestation by Strongyloidea.

Samples of ewe faeces were taken from the rectum at intervals throughout the experiment: the egg contents were determined by the use of a modified 'McMaster technique' (Morgan, Parnell & Rayski, 1950) but in which counts of two, instead of four, cells were made.

The ewes were randomly divided on 11 May into three groups, designated (N + K), K, and control groups, consisting respectively of nine, six and six animals together with their lambs. For the next 10 days two of the groups received perennial rye-grass from plots fertilized with potash and nitrogen (N + K group) and with potash only (K group), the control group continuing throughout on the original diet. The grass, cut daily at 9 a.m., was given to the ewes as soon as possible after collection. It was fed to appetite and was taken readily by ewes and lambs in both groups. Consumption per head of grass from the nitrogen-treated sward, however, was higher than of grass from the other sward. At the end of the 10-day period the grass-fed animals returned to the original diet, and all animals then continued on this until observations stopped on 12 June.

Samples of whole blood, collected into Wintrobe's oxalate mixture, were removed from the jugular vein of each ewe immediately prior to and at intervals during the grass-feeding period. A total of 350 ml. was removed from each animal, 50 ml. being taken seven times at 2-day intervals. (These quantities were required for concurrent chemical estimations unrelated to the present purpose.) Determinations of P.C.V. were made on four occasions, by spinning the blood in haematocrit tubes at an R.C.F. of 2760 G. at 25° C. for 90 min., on the day of collection.

In Expt. 2 no bleedings were carried out, and no cut grass was fed: observations were confined to the course of faecal worm-egg counts. A group of ninteen lambs (Blackface × Greyface, born March–April 1958), containing thirteen ewe-lambs and six wethers was kept in the field until mating time. The animals, from now on described as hoggs, were then divided. On 27 October seven ewe-hoggs were transferred to another field into which a tup was introduced. The wether-hoggs were also removed to this field on account of the shortage of feed in the original field in which the other six ewe-hoggs remained. Both fields were old grass heavily grazed by sheep.

The animals were brought together again on 28 November, and kept in a Dutch barn until 23 December when they were transferred to a large bay in a totally enclosed sheep house. A temporary lambing pen was constructed in the bay in

mid-March for the five ewe-hoggs which had become pregnant: at the end of the lambing period it was dismantled. (Lambing details are given in Table 3.)

The animals were maintained on a daily ration of approximately 1 lb. of ewe-nuts (B.O.C.M.) with unlimited hay throughout the whole period of living indoors.

The system described above for prevention of strongyloid reinfestation was also used here, except that caustic-soda treatment was applied twice as frequently. Its success was established by failure to demonstrate faecal worm-eggs in samples from a ewe-hogg kept under these conditions from birth, which was added to the group at the date of entry to the enclosed sheep house, and which remained with them until observations were discontinued. The four surviving lambs continued free of strongyloid worms up to 11 May, when they were removed for other purposes; eggs of *S. papillosus*, however, were seen in the faeces of two of them.

Egg counts, by the method described above, were made at approximately fortnightly intervals up to the end of April, and thereafter at approximately weekly intervals until the end of the experiment.

The animal groups in this experiment are summarized as follows:

(A) five primiparous ewe-hoggs; (B) two nulliparous ewe-hoggs; (C) six wether-hoggs; (A, B and C having identical history regarding exposure to worm infestation); and (D) six nulliparous ewe-hoggs (of different exposure history during the month (November) preceding transfer to Dutch barn).

RESULTS

Experiment 1

When the ewes were brought indoors in early February their mean faecal egg count was low (95 ± 54 eggs/g.). There was no notable change from this level for the next 8 weeks. Towards the end of April, after all the animals had lambed, the egg counts began to rise rapidly to reach a mean value of 4270 ± 848 eggs/g. in mid-May. The course of this rise, and its relation to the periods of lambing and grass feeding are shown in Fig. 1. The mean increase continued through the grass-feeding period, between the beginning and end of which the difference was highly significant ($P < 0{\cdot}01$).

Individual egg counts varied considerably: the spring-rise was not observed in all the animals (Fig. 2), nevertheless, it reached very significant dimensions in the majority. It is impossible with these data to define precise temporal limits to the spring-rise, but Fig. 2 shows a similarity of pattern for most of the individuals, the period of rapid increase in egg count occurring some 3–4 weeks after lambing. There is no evidence that grass-feeding affected the numerical course of the worm-egg output either directly or through changes in faeces consistency.

The mean values of P.C.V. of the three groups of ewes are given, together with their concurrent mean faecal egg counts in Table 1. This shows a fall in P.C.V., and a rise in egg count in all three groups during the grass-feeding period. Analysis of variance of the P.C.V. data (Table 2) establishes the significance of the falling values ($P < 0{\cdot}05$) but gives no significance to the differences between the groups.

Logarithmic transformation of faecal egg-count data has been shown to be

efficient for purposes of statistical analysis by Paver, Parnell & Morgan (1955). A transformation, $x = \log(N/100+1)$, where N is the number of eggs/g. of faeces, was employed, therefore, and a close, highly significant negative correlation was obtained between x and P.C.V., namely, $r = -0{\cdot}77$ ($P < 0{\cdot}01$).

From covariance analysis (Table 2), using the transformed egg-count data as the x variable, the adjusted means for the P.C.V. of the three groups of animals over the 10-day period are found to be: (N+K 31·2, K 29·2 and control 29·0. The

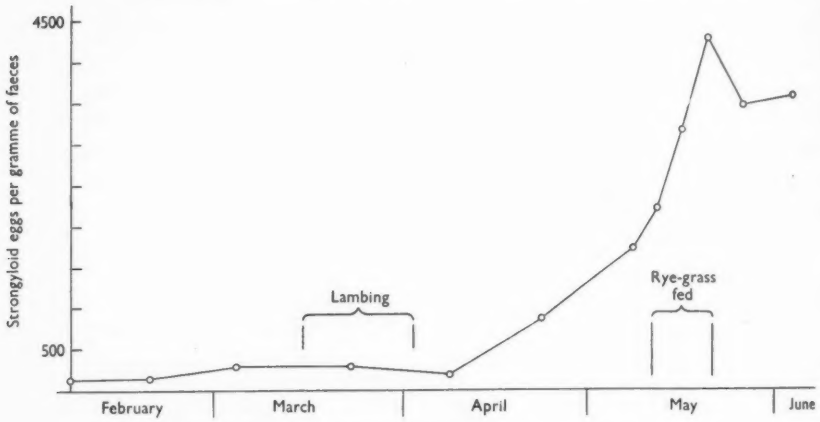


Fig. 1. Mean spring rise in relation to lambing times and the grass-feeding period (Expt. 1).

Table 1. *Mean* packed red-cell volume* (%) *and mean* worm-egg count* (*hundreds/gramme of faeces*) *of the three groups during the grass-feeding period*

Date of sampling	Days on grass	Group (N+K) (9 ewes)		Group K (6 ewes)		Control group (6 ewes)	
		Worm eggs	P.C.V.	Worm eggs	P.C.V.	Worm eggs	P.C.V.
9. v. 59	−1	17·8± 4·5	31·4±1·1	18·7± 9·2	30·9±2·0	15·2±7·0	30·8±1·3
12. v. 59	2	27·2± 9·7	31·4±1·0	20·5± 9·6	32·0±2·0	15·8±5·0	30·7±1·4
16. v. 59	6	44·2±13·4	29·9±1·2	25·5±10·4	28·9±1·7	19·0±5·9	28·7±1·3
20. v. 59	10	45·2± 9·6	28·6±1·0	57·7±25·1	27·8±2·6	23·8±6·6	27·9±1·3

* With its standard deviation.

Table 2. *Analysis of variance and covariance of transformed egg count* (x) *and packed red-cell volume* (y) (*Cochran & Cox*, 1957, *cap.* 3·86)

Source of variation	D.F.	x^2	xy	y^2	Residuals D.F.	Residuals S.S. $(y-cx)^2$	Residuals M.S.	F
Diet	2	1·17	2·87	10·01	2	92·28	46·14	8·65 ($P < 0{\cdot}01$)
Bleeding	3	1·89	−16·38	144·70*	3	13·44	4·48	0·84 N.S.
Interaction (diet × bleeding)	6	0·23	0·07	6·02	6	16·10	2·68	0·50 N.S.
Error	72	17·65	−110·91	1075·76	71	378·74	5·334	—

* $F = 3{\cdot}23$, $P < 0{\cdot}05$.

difference between the first and the other two groups is highly significant ($P < 0{\cdot}01$). At the same time the adjusted means for the P.C.V. of all the ewes for the four sampling occasions are successively 30·2, 30·6, 29·6 and 29·5. Although a small downward trend is still evident it is statistically without significance.

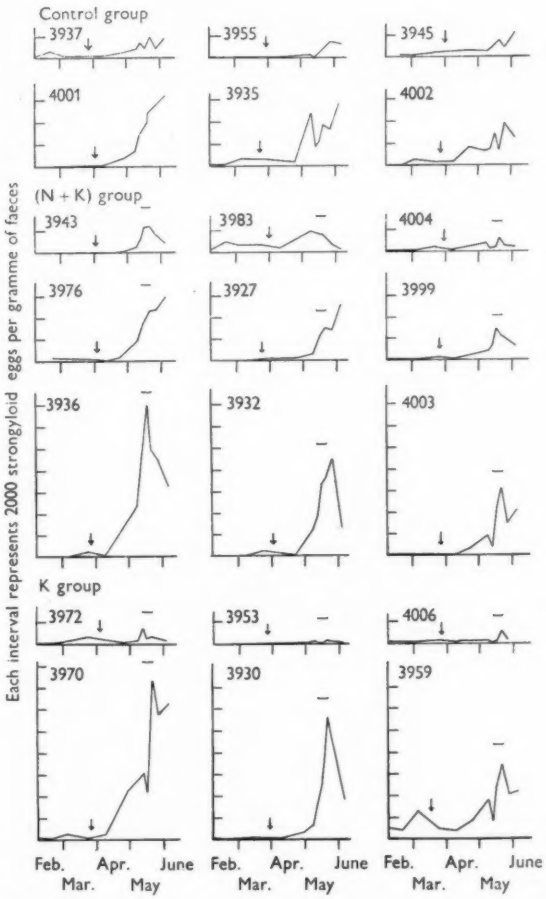


Fig. 2. Course of individual egg counts in Expt. 1. Lambing date is shown by arrow and grass-feeding period by horizontal line.

Experiment 2

The results of this experiment can be most readily appreciated by reference to the protocols given in Table 3. The experimental period was divided, with arbitrary reference to the pregnant animals, into three phases, namely: (1) pre-parturient, including the five egg-counting occasions up to 12 March; (2) parturient, including the April and 1 May counts, and (3) post-parturient, including the five occasions between 11 May and 17 June.

When the animals were brought in their egg counts were quite low, the mean for the nineteen animals on 14 January being 284 ± 61, and there was no evident tendency for the counts to rise during the first phase of observations. Analysis of

Table 3. *Egg counts (hundreds/gramme faeces) of hoggs in Expt.* 2

		Phase 1					Phase 2			Phase 3				
	Date...	14. i.	28. i.	12. ii.	25. ii.	12. iii.	1. iv.	17. iv.	1. v.	11. v.	20. v.	28. v.	10. vi.	17. vi.
Group A	5096*a*	3	1	1	1	1	0	0	1	4	6	7	9	18*
	5100*b*	8	2	2	1	1	4	2	3	6	3	1	3	5
	5103*c*	1	3	8	9	8	11	10	62	94	54	73	93	22*
	5111*d*	4	3	3	4	0	6	7	5	166	180	236	236	480*
	5114*e*	2	13	14	5	7	10	12	11	10	10	9	14	18
Group B	5102	9	2	7	6	5	1	12	5	5	3	0	10	11
	5109	3	1	0	0	0	3	4	3	4	7	2	5	8*
Group C	5097	7	1	2	0	0	0	0	0	0	0	0	0	0
	5098	0	0	2	9	4	0	1	1	4	7	5	10	13*
	5099	3	3	3	9	7	1	9	13	15	8	3	6	7
	5107	1	0	0	0	0	0	0	0	0	0	0	0	0
	5113	1	2	0	0	0	1	2	2	1	0	1	3	2
	5115	1	0	0	1	0	1	1	5	5	4	3	0	5**
Group D	5101	2	0	0	0	2	0	1	1	2	1	1	1	2
	5104	1	1	1	1	2	1	0	0	0	0	1	3	1
	5106	3	3	1	0	0	0	0	0	0	0	0	0	0
	5108	1	2	0	1	1	1	2	2	1	2	6	3	2
	5116	4	2	0	0	0	2	1	1	0	0	0	0	0
	5117	0	0	0	0	0	0	0	0	0	0	0	0	0
Control	5078	0	0	0	0	0	0	0	0	0	0	0	0	0

a, twin lambs born 28. iii. (one died 29. iii). *b*, single lamb born 15. iv. *c*, single lamb born 3. iv. *d*, single lamb born 1. iv. Ewe died 18. vi. *e*, single lamb born 14. iv. (died 12. v.).

* Rise significant $P < 0{\cdot}01$.

** Rise significant $P < 0{\cdot}05$.

the counts on the five occasions comprising this phase established the significance ($P < 0{\cdot}01$) of the higher counts, in the pregnant ewe-hoggs (group A). Between the other two groups (C and D), however, there was no significant difference during this period. The higher level of egg output in these ewe-hoggs became heavily emphasized in the third phase of the experiment, a very marked spring, or post-parturient rise having taken place in them. The mean counts for the third phase were 7032, 325, and 87 in groups A, C and D, respectively, as compared with first phase means of 420, 187 and 97. The spectacular differences between the courses of the group means are shown in Fig. 3. This treatment, however, obscures some of the evidence obtained. For example, the size of the rise in group A was determined largely by the output of animal number 5111 which succumbed to its heavy infestation on 18 June. The following worms were recovered at autopsy of this animal:

Haemonchus contortus—8500, *Trichostrongylus axei*—2150, *Ostertagia circumcincta*—650, *Trichostrongylus colubriformis* and *vitrinus*—750, and small numbers of *Nematodirus filicollis*, *Bunostomum trigonocephalum*, *Strongyloides papillosus*, *Oseophagostomum venulosum*, *Chabertia ovina* and *Trichuris ovis*.

Using the F test, to compare the differences between individual first- and third-phase counts, the results were:

Group	No. of animals	No. of animals in which increase was significant	
A	5	3	($P < 0{\cdot}01$ in each case)
B	2	1	($P < 0{\cdot}01$)
C	6	2	($P < 0{\cdot}05$, $P < 0{\cdot}01$)
D	6	0	—

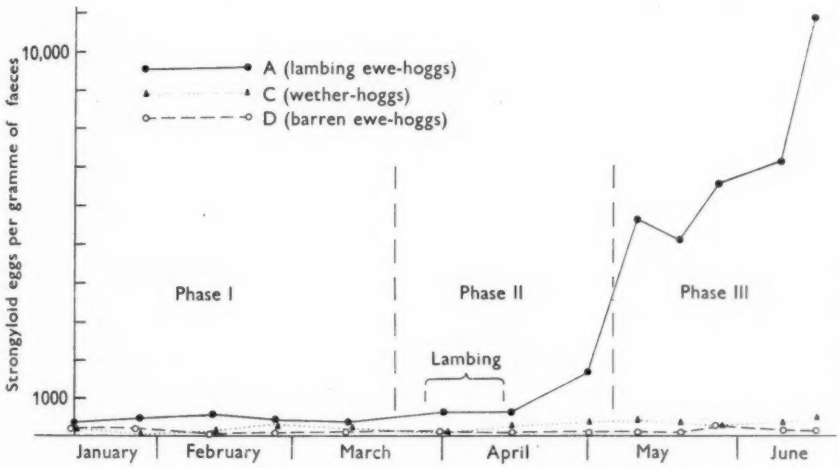


Fig. 3. Course of mean egg counts in three groups in Expt. 2.

DISCUSSION AND CONCLUSIONS

The findings of the first of the present experiments repeat the earlier experience of a fall in P.C.V. during the feeding of rye-grass to ewes previously maintained on hay and concentrates. On this occasion, however, the close inverse correlation obtained between P.C.V. and transformed faecal egg counts suggests that worm burden is a major factor. An inverse relationship between worm egg counts and erythrocyte levels has been demonstrated by various workers, including Andrews (1942), Hawkins, Cole, Kline & Drudge (1944), Hawkins & de Freitas (1947), and Grunsell (1955).

When that part of the variance in P.C.V. associated with the faecal egg counts is eliminated by covariance analysis, the adjusted mean of one of the groups (N + K) is significantly greater than those of the other two groups, although analysis of variance had revealed no difference between them. From this it follows that, not only is there no causal relationship between the change in diet and the falling values observed in P.C.V., but that the fall in one of the groups (N + K) was partially reduced by the feeding of its particular type of rye-grass. The phenomena here

described are, therefore, distinct from those involved in Jamieson's (1958) work, in which grazing of a mixture of rye-grass and clover produced a fall in P.C.V.

The experiment was not sufficiently comprehensive in design for a proper consideration of haemodilution as a possible causal factor in the reduction of P.C.V. Nevertheless, from the absence of a statistically significant trend in the adjusted means of all the animals on the successive sampling occasions, it follows that, either the blood-letting at sampling had no significant effect, or if sampling produced any part of the observed anaemia it was not an independent factor. Holman & Pattison (1941) and Holman (1945) have shown that the anaemia associated with *Haemonchus contortus* infestation is not entirely of post-haemorrhagic type, but may be partly dyshaemopoietic and of alimentary origin, although Fourie (1931), Martin & Clunies Ross (1934), Andrews (1942) and others consider that the primary factor is direct blood loss. We may conclude, therefore, that the falls in P.C.V. observed in this experiment were either entirely and directly due to the worm infestations, or were due to impairment, in part at least, by the worm infestations of recovery from the loss of blood sustained through sampling. In view of the comparatively small quantities of blood removed at sampling, it seems reasonable to prefer the former alternative.

The most interesting feature of the present experiments was the occurrence in housed sheep, both 3-year-olds and hoggs, of spring-rises in faecal egg count of magnitudes comparable to any that have been described under field conditions. The phenomenon has been observed in housed animals by other workers, among whom Hawkins *et al.* (1944) assumed the rise to have been due to reinfestation upon the advent of warm weather, infective larvae having developed from eggs produced by the existing small populations. In other cases reinfestation appears to have been minimal or absent. Thus, Hawkins & de Freitas (1947) concluded that the rising egg counts they observed prior to April could not, on account of low temperature, have been due to reinfestation. Naerland (1952), states that 'no actual reinfestation was proved' while his animals were housed, and Spedding & Brown (1956), like ourselves, took active measures to prevent reinfestation in their houses.

Taylor (1935), who appears to have been the first worker to describe the spring-rise in sheep, emphasized the fact that it did not coincide with the period of maximum availability of infective larvae. Cushnie & White (1948) were also led by this fact to argue that, if an increase in worm population were an essential preliminary to increased egg output, the peak output of eggs would occur later in the year. To account for an increased egg output independent of, or occurring in the probable absence of uptake of infective larvae Taylor (1935), Hawkins & de Freitas (1947), Cushnie & White (1948) and Naerland (1952) all postulated a probable increased fecundity of the existing adult population, although the last-named author also considered possible an augmentation of the adult population through maturation of a latent larval population. Taylor further quoted, as evidence to support the hypothesis, an observation that trichostrongylid worms could produce up to twelve times more eggs in susceptible than in resistant lambs. Taylor (1934) also gave evidence of very variable ratios between adult numbers of *Trichostrongylus*

spp. and faecal egg counts. Soulsby (1957*a*) observed a small rise in egg count occurring concurrently with a fall in antibody titre which could indicate the possibility of an increase in egg production by the existing adult population. This, however, did not initiate the 'spring-rise', since the main egg-count rise was delayed for a further 2 weeks, and was not fully established until 4–6 weeks after the reduction in titre.

The hypothesis of varying fecundity depends mainly upon negative evidence, and apart from Taylor's observations and Soulsby's deduction lacks direct support. When it was first put forward it appeared to be the only mechanism likely to account for changes in egg output in the absence of infective larvae. The theory, which remained unchallenged until Naerland (1952) suggested the possibility of latent larvae developing to the adult stage, would have been greatly strengthened by a demonstration of suppression of the spring-rise by winter anthelminthic treatment. There appears to be no such evidence, but on the contrary Hawkins *et al.* (1944) Seghetti & Marsh (1945), and Naerland (1952) have all observed spring-rise following previous anthelminthic treatment.

Morgan, Parnell & Rayski (1951) slaughtered a series of hill sheep at intervals throughout the year and found the adult worm populations to vary concurrently with the previously described variations in faecal egg counts (Morgan *et al.* 1950 and Morgan & Sloan, 1947). Worm burdens were much heavier at the time of spring-rise than at other times and, moreover, larval populations in the gut lumen were maximal at this period. These workers concluded that the variation in number of adult worms adequately accounts for the variations observed in faecal egg counts without the need to postulate a seasonal variation in fecundity. Morgan and his co-workers (1951) failed to find evidence of overwintering of latent worm larvae in the gut mucosa and concluded that the increased spring worm-burdens were derived from recently acquired infective larvae. This conclusion, however, would imply that the spring-rise observed by Morgan and his co-workers was different from those observed by the workers whose experimental conditions had precluded ingestion of infective larvae, or from those described in low-ground sheep by Crofton (1954). Crofton has drawn attention to the individual variation which occurs in the temporal incidence of spring-rise, and has described individual peaks occurring within one flock at dates between February and June. The relatively constant interval between individual dates of lambing and of maximal faecal egg count in his work indicates strongly an intrinsic origin for the rise, and its independence of the availability of infective larvae. Crofton (1958) has also described a post-parturient rise in autumn-lambed ewes with the same characteristics as the post-parturient or spring-rise observed in spring-lambed ewes.

To reconcile his findings with those of Morgan *et al.* (1951), Crofton (1954) advanced a modified theory of larval uptake. He postulated a period of declining immune status during which there is a slow accumulation of larvae 'which do not necessarily invade and remain in the mucosa'. A development *en masse* occurs after the immune status has fallen below a critical level and the worms reach patency simultaneously.

In the present experiments the development of adult populations from newly

acquired larval infestations was prevented for periods of from 3 to 5 months before the occurrence of the increased egg output and, therefore, the theories of Morgan *et al.* (1951) and Crofton (1954) are inapplicable. The magnitude of the rises observed, and the magnitude of the worm population recovered after death from individual 5111 preclude acceptance of what seems to us the aready tenuous hypothesis of enhanced fecundity of extant adults. The conclusion is then compelled that the increase in egg output is due to augmentation of the mature population by individuals derived from a latent larval population acquired some months previously. Nevertheless, Spedding & Brown (1956), whose management was similar to our own, rejected this view on the dubious argument that from August to March is too long a period for latency, and adhered to the idea of increased fecundity of adults which in their experiments must have survived multiple dosing with phenothiazine.

Some strongyloid species such as *Haemonchus contortus* are reported to have a brief and relatively invariable histotropic phase in their parasitic development (Stoll, 1943), while others exhibit great variation in the duration of this phase, for example, *Ancylostoma caninum* (Scott, 1928) and *Ostertagia circumcincta* (Threlkeld, 1934). A prolonged delay in the histotropic phase has been known, since Veglia's (1924) work on *Oesophagostum columbianum*, larvae of which can be identified in the intestinal mucosa months after being acquired. More recently, inhibition of development in the histotropic phase has been described in *Oesophagostomum dentatum* and *Hyostrongylus rubidus* of the pig by Kotlán (1952), in *Trichostrongylus retortaeformis* of the rabbit by Michel (1952), in *Trichonema* spp. of the horse by Gibson (1953), and in *Cooperia curticei* and *Ostertagia circumcincta* of the sheep by Andrews (1939) and Sommerville (1954), respectively.

Kotlán (1952) refers to uninterrupted development in the histotropic phase as regular, and to inhibited development as irregular, and he assumes that inhibited histotropic forms are unlikely to resume and complete their development. Sommerville (1954) has indeed described disintegration among larvae of *Ostertagia circumcincta* whose period of inhibition had exceeded 8 weeks. That this is not the inevitable fate of all, however, is to be deduced from the observations of Gibson (1953) on *Trichonema* spp. and Michel (1952) on *Trichostrongylus retortaeformis*.

Associated with inhibited development there is frequently a tissue reaction resulting in nodule formation on the part of the host. Mönnig (1934) first drew attention to the relation between host resistance and nodule formation, and it is now generally agreed that inhibition of larval development is a manifestation of host resistance (Andrews, 1939; Sarles, 1944; Taylor & Michel, 1953).

Although inhibited development is known in some sheep strongyloids, latency such as that found in *Trichonema* spp. and *Trichostrongylus retortaeformis* has not yet been demonstrated. Nevertheless, the present evidence is strongly indicative of its probable occurrence. The findings of Stewart (1950), who demonstrated an inverse relationship between humoral antibody titre and egg count, and of Soulsby (1956, 1957*a*) who showed a seasonal fluctuation in antibody levels, with low levels preceding the spring-rise period, provide a basis to account for inhibition and resumption of larval development at the appropriate periods. It is true that the results of these workers also form a basis for Crofton's (1954) theory, but it is conclusive from

the present findings that, while permissive, the gradual accumulation of larvae postulated by him is not an obligatory preliminary to spring-rise.

The main difficulty opposing acceptance of the latent larva hypothesis is found in the failure of Morgan *et al.* (1951) and Soulsby (in reply to discussion on Soulsby, 1957*b*) to identify latent larvae in winter autopsies. On the other hand, Sommerville (1954) has difficulty in reconciling these reports with his own field findings in Australia of inhibited *Ostertagia circumcincta* larvae in naturally infected sheep. This species is one of the most prominent contributors to the spring-rise in British lowland (Crofton, 1954) and upland sheep (Morgan *et al.* 1951).

The present experiments support Crofton's (1958) observation that although spring-rise may occur in non-reproducing hosts it is less common and much less pronounced than in breeding ewes. We may conclude, therefore, that the present work strongly supports the view that spring-rise in faecal egg count is an indication of the maturation of latent overwintering larvae, this being a sequel to a decline in the immune status of the host. The phenomenon may be enhanced by stress factors such as parturition (Crofton, 1954), severe climate and malnutrition (Paver *et al.* 1955) and may be alleviated by improved diet (White & Cushnie, 1952). To the species already known to exhibit latency there is a probability that *Trichostrongylus axei* and *Haemonchus contortus* will have to be added in spite of Stoll's (1943) finding of constancy in the duration of the histotropic phase of the last named.

The present findings provide a caveat, especially to those concerned with nutritional problems in breeding ewes. A fall in P.C.V. to levels associated with loss of body weight (Jamieson, 1958), and even a fatal helminthiasis can occur months after acquisition of a worm infestation. Not only can these effects modify results but the individual variability of the spring-rise phenomenon, and its probable occurrence in spite of anthelminthic dosing can seriously impair the design and interpretation of experiments concerned with other topics.

SUMMARY

1. A spring-rise in faecal strongyloid egg count is described in sheep housed under conditions designed to prevent reinfestation. Large rises were observed in the majority of 3-year-old breeding ewes and primiparous ewe-hoggs, and occasional small rises occurred in nulliparous ewe-hoggs and wether-hoggs.

2. Falls were observed in the packed red-cell volumes of ewes during the period of spring-rise and a close inverse correlation was found between packed-cell volumes and transformed worm egg counts. No evidence was obtained to attribute the falls in packed-cell volume to factors other than worm burden.

3. The fall in packed-cell volume was partially alleviated by feeding of one type of perennial rye-grass, but no direct effect of feeding on the worm-egg output was found.

4. Theories of spring-rise are discussed and the conclusion reached that latent overwintering larvae must play a major part generally in the phenomenon.

Our thanks are due to Mrs Anna Smart and Mr J. E. Patterson for technical assistance, and to Messrs J. Gibson and I. MacLeod for carrying out the system of animal management.

REFERENCES

ANDREWS, J. S. (1939). Life history of the nematode *Cooperia curticei*, and development of resistance in sheep. *J. Agric. Res.* **58**, 771–85.

ANDREWS, J. S. (1942). Stomach worm (*Haemonchus contortus*) infection in lambs and its relation to gastric haemorrhage and general pathology. *J. Agric. Res.* **65**, 1–18.

COCHRAN, W. G. & COX, G. M. (1957). *Experimental Designs*. 2nd edition, xiv and 611 pp. New York and London.

CROFTON, H. D. (1954). Nematode parasite populations in sheep on lowland farms. I. Worm egg counts in ewes. *Parasitology*, **44**, 465–77.

CROFTON, H. D. (1958). Nematode parasite populations in sheep on lowland farms. V. Further observations on the post-parturient rise and a discussion of its significance. *Parasitology*, **48**, 243–50.

CUSHNIE, G. H. & WHITE, E. G. (1948). Seasonal variations in faeces worm-egg counts of sheep. *Vet. Rec.* **60**, 105–7.

FOURIE, P. J. J. (1931). The haematology and pathology of haemonchosis in sheep. *Rep. Vet. Res. S. Afr.* **17**, 495–572.

GIBSON, T. E. (1953). The effect of repeated anthelminthic treatment with phenothiazine on the faecal egg counts of housed horses, with some observations on the life cycle of *Trichonema* spp. in the horse. *J. Helminth.* **27**, 29–40.

GRUNSELL, C. S. (1955). Seasonal variation in the blood and bone marrow of Scottish hill sheep. *J. comp. Path.* **65**, 93–107.

HAWKINS, P. A., COLE, C. L., KLINE, E. E. DRUDGE, J. H. (1944). Studies of sheep parasites. I. The course of untreated nematode infections. *Vet. Med.* **39**, 154–161.

HAWKINS, P. A. & DE FREITAS, M. G. (1947). Studies of sheep parasites. VIII. Overwintering of nematode larvae. *J. Parasitol.* (Suppl.) **33**, 22.

HOLMAN, H. H. (1945). Studies on the haematology of Sheep. V. A survey of blood pictures in sheep diseases. *J. Comp. Path.* **55**, 229–42.

HOLMAN, H. H. & PATTISON, I. H. (1941). A study of the blood picture of lambs suffering from parasitic gastritis. *Vet. Rec.* **53**, 491–8.

JAMIESON, N. D. (1958). Adverse effect of nitrate metabolic products on sheep growth. *Nature, Lond.*, **181**, 1601–2.

KOTLÁN, A. (1952). The development and pathological significance of the histrotopic (*sic.*) phase in parasitic nematodes. *Rep.* 14*th Int. Vet. Congr.* **2**, 61–4.

MARTIN, C. J. & ROSS, I. CLUNIES (1934). A minimal computation of the amount of blood removed daily by *Haemonchus contortus*. *J. Helminth* **12**, 137–42.

MICHEL, J. F. (1952). Inhibition of development of *Trichostrongylus retortaeformis*. *Nature, Lond.*, **169**, 933–4.

MÖNNIG, H. O. (1934). *Veterinary Helminthology and Entomology*, xvi + 402 pp. London.

MORGAN, D. O., PARNELL, I. W. & RAYSKI, C. (1950). Further observations on the seasonal variation in worm egg output in Scottish hill sheep. *J. Helminth.* **24**, 101–22.

MORGAN, D. O., PARNELL, I. W. & RAYSKI, C. (1951). The seasonal variations in the worm burden of Scottish hill sheep. *J. Helminth.* **25**, 177–212.

MORGAN, D. O. & SLOAN, J. E. N. (1947). Research on helminths in hill sheep with special reference to seasonal variations in worm-egg output. *Scott. Agric.* **27**, 28–35.

NAERLAND, G. (1952). Nutrition in relation to nematode parasitism in sheep. *Rep.* 14*th Int. Vet. Congr.* **2**, 65–70.

PAVER, H., PARNELL, I. W. & MORGAN, D. O. (1955). Some factors influencing the seasonal variation in worm egg counts in Scottish hill sheep. *J. comp. Path.* **65**, 220–35.

SARLES, M. P. (1944). Effects of experimental nodular worm (*Oesophagostomum columbianum*) infection in sheep. *U.S. Dept. Agric. Tech. Bull.* **875**, 19 pp.

SCOTT, J. A. (1928). An experimental study of the development of *Ancylostoma caninum* in normal and abnormal hosts. *Amer. J. Hyg.* **8**, 158–204.

SEGHETTI, L. & MARSH, H. (1945). Control of intestinal parasitism in lambs by winter treatment of ewes, as compared with the use of phenothiazine in salt in summer. *Amer. J. Vet. Res.* **6**, 159–64.

SOMMERVILLE, R. I. (1954). The histotropic phase of the nematode parasite, *Ostertagia circumcincta*. *Aust. J. Agric. Res.* **5**, 130–40.

SOULSBY, E. L. J. (1956). Studies on the serological response in sheep to naturally acquired gastro-intestinal nematodes. I. Preparation of antigens and evaluation of serological techniques. *J. Helminth.* **30**, 129–42.

SOULSBY, E. L. J. (1957*a*). Studies on the serological response in sheep to naturally acquired gastro-intestinal (*sic*) nematodes. II. Responses in a low ground flock. *J. Helminth*, **31**, 143–60.

SOULSBY, E. L. J. (1957*b*). Some immunological phenomena in parasitic infections. *Vet. Rec.* **69**, 1129–39.

SPEDDING, C. R. W. & BROWN, T. H. (1956). The 'spring rise' in the nematode egg-count of sheep. *J. Helminth* **31**, 171–8.

STEWART, D. F. (1950). Studies on resistance of sheep to infestation with *Haemonchus contortus* and *Trichostrongylus* spp. and on the immunological reactions of sheep exposed to infestation. IV. The antibody response to natural infestation in grazing sheep and the 'self cure' phenomenon. *Aust. J. Agric. Res.* **1**, 427–39.

STOLL, N. R. (1943). The wandering of *Haemonchus* in the sheep host. *J. Parasit.* **29**, 407–16.

TAYLOR, E. L. (1934). A method of estimating the number of worms present in the fourth stomach and small intestine of sheep and cattle for the definite diagnosis of parasitic gastritis. *Vet. Rec.* **14**, 474–6.

TAYLOR, E. L. (1935). Seasonal fluctuation in the number of eggs in the faeces of ewes. *J. Parasit.* **21**, 175–179.

TAYLOR, E. L. & MICHEL, J. F. (1953). The parasitological and pathological significance of arrested development in nematodes. *J. Helminth* **27**, 199–205.

THRELKELD, W. L. (1934). The life history of *Ostertagia circumcincta*. *Bull. Va. Agric. Exp. Sta. Tech.* **52**, 21 pp.

VEGLIA, F. (1924). Preliminary notes on the life-history of *Oesophagostomum columbianum*. *9th and 10th Rep. Dir. Vet. Educ. and Res. Dep. Agric. S. Afr.*, April 1923, pp. 811–823.

WHITE, E. G. & CUSHNIE, G. H. (1952). Nutrition and gastro-intestinal helminths in sheep on hill grazing: The effect of a dietary supplement on faecal worm-egg counts, worm burden, body-weight and wool production. *Brit. J. Nutr.* **6**, 376–86.

Parasitology (1960), **50**, 401–417
With 1 plate
Printed in Great Britain

The larval trematodes of certain fresh-water molluscs

II. Experimental studies on the life-cycle of two species of Furcocercariae

By CHRISTINE ILES, Ph.D.

*Joint Fisheries Research Organization, P.O. Nkata Bay, Nyasaland**

(*Received 22 October* 1959)

During the course of a 3-year study the life cycles of several species of furcocercariae were investigated. *Cercaria paracauda*, Iles, was observed to penetrate into fish and develop within the lens into a diplostomulum type metacercaria. *C. Apatemon gracilis minor*, Yamaguti syn. *C. hamburgensis* (Komiya, 1938) and *C. tetraglandis* (Iles, 1959) were found to penetrate into fresh-water leeches and develop into tetracotyle metacercariae. The diplostomulum and tetracotyle metacercariae may easily be differentiated on the basis of the presence or absence of a cyst wall and the structure of the reserve bladder system (Komiya, 1938). The development of the metacercariae of *C. A. g. minor* and *C. tetraglandis* within the leech intermediate host was followed. It was possible to trace the development of the adult trematode *A. g. minor*, Yamaguti, in laboratory-reared ducklings which were force-fed with experimentally infected leeches.

Previous to this study Szidat (1924, 1929), Harper (1931) and Komiya (1938) had attempted to follow the development of tetracotyle metacercariae in some detail. Szidat investigated the development of the metacercariae of *Apatemon gracilis gracilis*, Rud. and *Cotylurus cornutus*, Rud. The development of the latter species was also studied by the other two authors. Harper alone, however, attempted to follow the development systematically by a series of dated experiments, but he made relatively few observations. Szidat and Komiya merely observed differences between forms at varying stages of development and presumably at different ages. It was noted during the present study that the development of the metacercariae within the same host and resulting from a single infection was not simultaneous, even though penetration by all the cercariae was completed within a few hours. Metacercariae of approximately the same age were found to be at markedly different stages of development.

Seven species of tetracotyle metacercariae have been recorded from fresh-water leeches; they are *Tetracotyle hirudinum*, Diesing (Hughes, 1928); *T. bdellocystis* (Lutz, 1921); *T. Apatemon intermedius* (Johnston & Beckwith, 1947 and Johnston & Angel, 1951); *T. A. gracilis gracilis* (Szidat, 1929, 1931), *T. A. g. burti* (Stunkard, Willey & Rabinowitz, 1941); *T. Cotylurus cornutus* (Szidat, 1929) and *T. dubia* (Wisniewski, 1935). The first two species have not been described sufficiently to allow a detailed comparison with those studied during this investigation. The

* Present address: Zoology Department, University College, Cardiff.

remaining five species may be differentiated on the basis of size, biology and the structure of the related cercariae (Table 1). It would be very difficult to separate these metacercariae on the basis of morphology alone without a knowledge of their biology and related cercariae.

METHODS OF STUDY

The leeches used in the life-cycle experiments were obtained from Cleabarrow Tarn at Windermere. Before being infected with cercariae these leeches were examined under a binocular microscope after slight compression between two glass slides. In this way the presence of a natural infection with either unencysted or encysted forms was readily discerned and any infected leeches were not used. Similarly, the degree and stage of infection was determined in experimentally infected leeches before they were fed to laboratory-reared ducklings. Specimens of *Erpobdella testacea* (Sav.) were used in the life-history experiments, although *Glossiphonia complanata*, L. and *Protoclepsis tesselata*, Müll were used in host specificity tests. The leeches were infected by placing them in a dish containing a concentrated suspension of recently emerged cercariae.

The final hosts used were laboratory-bred rats and Khaki Campbell ducklings which were not fed before infection nor for a period of 12 hr. after infection. They were force-fed with living leeches either entire or in sections if the leeches were large. With and after each administration of infective material, water was pipetted into the mouths of the animals in an attempt to ensure that the material was swallowed and not immediately regurgitated. Each animal was isolated after infection, and if regurgitation occurred it was noted. Observations were made on metacercariae resulting from experimental infection only and obtained, by dissection with fine needles, from living leeches. These specimens were examined alive and some were fixed in hot 10% formalin. The measurements of the metacercariae at different ages were taken from ten living and ten fixed specimens, except where otherwise stated.

The histological studies were made on experimentally infected leeches which had been narcotized in chloroform-water and then fixed in Zenker's fluid. This fixative was found to be suitable for both the tissues of the parasite and the host. Sections were cut at 10μ and stained in Weigert's haematoxylin and Ponceau S. Observations were also made on living and fixed specimens and sectioned material of the adult trematode *Apatemon gracilis minor*. The adults were removed from the intestine of the host, placed in warm-blooded Ringer solution maintained at a temperature of 40° C. and carefully freed from mucus and debris. Measurements of the adults at different stages of development were taken from ten living specimens and ten specimens fixed in hot 10% formalin, stained in aceto-carmine and mounted in balsam. The adult trematode material used for sectioning was fixed in Bouin's fluid, stained in borax carmine to facilitate embedding in paraffin wax and cut at 10μ. The sections were treated with acid alcohol to remove the borax carmine and stained in Weigert's haematoxylin and Ponceau S.

Table 1. *A comparison of some tetracotyle metacercariae found in fresh-water leeches*

Table 1. *A comparison of some tetracotyle metacercariae found in fresh-water leeches*

Metacercaria	Measurements (mm.)	Distribution in host	Flame cell system of cercaria	Penetration gland cell system of cercaria
Tetracotyle dubia	L. 0·35–0·56 B. 0·25–0·46 O.S. 0·055–0·063 × 0·055–0·06 V.S. 0·066–0·077 × 0·066–0·077 Cyst wall 0·015–0·04	Digestive gland of snails and surface tissue of leech	2(3 + 2 + (2)) = 14	2 pairs anterior penetration gland cells
Metacercaria of *A. g. burti*	32 days L. 0·607 B. 0·451 Cyst wall 0·038 × 0·078 42 days L. 0·45 B. 0·295	Blood vascular system of Arhynchobdellid leeches	2(2 + 4 + (1)) = 14. Posterior excretory commissure	4 pairs posterior penetration gland cells
Tetracotyle C. cornutus	Mathias, 1925 L. 0·341–0·521 B. 0·260–0·412 O.S. 0·046–0·058 V.S. 0·062–0·073	Digestive gland and hermaphrodite organ (Mathias) of mollusc. Sex glands and blood vascular system of leech	2(4 + 4 + (2)) = 20. Anterior excretory commissure	2 pairs anterior penetration gland cells
Tetracotyle A. intermedius	23 days. Living L. 0·265 B. 0·206 Inner cavity of cyst 0·205 × 0·176 Fixed 0·158 × 0·113–0·225 × 0·165	Blood vascular system of Rhynchobdellid leeches	2(2 + 4 + (1)) = 14. Posterior and anterior excretory commissures	4 pairs posterior penetration gland cells
Tetracotyle A. g. gracilis	L. 0·44 B. 0·32 Cyst wall 0·05 × 0·08	Blood vascular system of Arhynchobdellid leeches	2(2 + 4 + (1)) = 14. Posterior excretory commissure	4 pairs posterior penetration gland cells
Tetracotyle A. g. minor	170 days. Fixed 10 % formalin. Outer diameter of cyst 0·32–0·38 × 0·24–0·28. Inner diameter of cyst 0·21–0·28 × 0·16–0·19	Leech tissue; not within blood vascular system	2(2 + 4 + (1)) = 14. Anterior blind ending ducts and posterior commissure	4 pairs posterior penetration gland cells

OBSERVATIONS ON THE PENETRATION OF CERCARIAE INTO FRESH-WATER LEECHES

The leeches, after being examined for infections, were placed in a vessel containing numerous recently emerged individuals of *Cercaria tetraglandis* or *C. A. g. minor* and exposed to infection for a few hours. Soon after their introduction the leeches showed signs of agitation, and either detached themselves from the walls of the receptacle and swam actively, or attached themselves to the bottom and exhibited strong writhing movements of the whole body. They exuded large quantities of mucus, presumably in an attempt to detach the cercariae and to prevent further numbers from penetrating. The penetration of the cercariae was readily observed under the low power of the microscope. The cercariae would attach themselves to the leech, then become detached and attack other places several times, until, by strong protrusions and contractions of the body, the anterior tip was finally inserted into the leech tissues. This initial penetration was presumably facilitated by the spines at the anterior extremity and by the secretion of the penetration gland cells. Following the insertion of the anterior part of the body, decaudation occurred and final penetration was accomplished relatively rapidly.

Several cercariae, however, which failed to penetrate after decaudation, became detached and were observed creeping on the bottom of the receptacle. Some of the cercariae that were attached to the posterior sucker of the leech were still attempting to penetrate 24–30 hr. after infection, although their activity was by then much reduced. The majority of these died before they could penetrate into the sucker. If very large numbers of cercariae were introduced, after 10–20 min. the leeches were covered with cercarial bodies, which formed a white hair-like investment, which was particularly obvious on the dorsal and lateral surfaces and readily visible to the naked eye. It is interesting to note that when a dead leech was introduced into a suspension of cercariae, they became attached to it, shed their tails, and attempted to penetrate while any other inert body did not elicit this response. This fact, together with the rapidity with which the living leech became infested with numerous cercariae, suggested that contact with the second intermediate host was not entirely a matter of chance facilitated by the activity of the leech, but that a specific chemotactic attraction may have existed between the two. Although the mucus secreted by the leech following the attack of the cercariae appeared to hinder penetration, nevertheless, a large proportion of cercariae succeeded in penetrating into the leech. The leeches appeared to be capable of withstanding very heavy infections without any apparent ill-effects and some heavily infected individuals were even found to reproduce and to deposit cocoons in laboratory cultures. Prolonged exposure of leeches to a large number of cercariae could, however, result in death.

The facts that *Cercaria tetraglandis* and *C. Apatemon g. minor* have an identical resting position, which is the same as that of *C. A. g. burti*, *C. A. g. gracilis*, *C. A. intermedius*, syn. *C. lessoni* and *C. Cotylurus cornutus* and that fresh-water

leeches are the secondary intermediate hosts for all these species, suggest that the resting position has some significance as an indicator of the type of life history and of the specific relationships of a cercaria.

THE DISTRIBUTION, ENCYSTMENT AND PATHOLOGY OF THE METACERCARIAE

Neither the metacercariae of *Cercaria tetraglandis* nor those of *Apetemon g. minor* localize within the blood vascular system as do those of *A. g. gracilis* (Szidat, 1931), *A. g. burti* (Stunkard, Willey & Rabinowitz, 1941) and *A. intermedius* (Johnston & Angel, 1951). Nor did they come to lie within the reproductive organs as do the metacercariae of *C. cornutus* (Szidat, 1929). Many of the metacercariae of *C. tetraglandis* occupied a superficial location within the body of the leech host, being situated dorsally and laterally within the surface musculature. Some metacercariae, however, were found within the parenchymatous and botryoidal tissue. The metacercariae of *A. g. minor* were found to be distributed throughout the whole body of the leech often in close proximity to, though not within, the blood vessels (Pl. I). These were readily distinguished by their contents and by the surrounding layer of botryoidal tissue.

In sections of infected leeches the encysted metacercariae appeared to be located in cavities in the body tissue (Pls. I, II) and there was a distinct, relatively wide gap between the margin of the cyst wall and the host tissue. This may have been an artifact produced by the differential shrinkage of the metacercariae and the well-muscularized host tissue. In Pl. II an encysted metacercaria of *A. g. minor* is shown with part of the cyst wall attached to the host tissue. If this was the result of a firmer attachment in this region which persisted throughout the fixation, embedding, staining and mounting procedures, then it is possible that there is normally a close attachment between the entire outer surface of the tetracotyle cyst wall and the tissue. The unencysted forms were surrounded quite closely by host tissue and were lying in cavities which followed approximately the shape of the metacercariae. Erasmus (1958) referring to *Diplostomulum X* suggested that a lytic substance was produced over the entire body surface by subcuticular gland cells. This would result in the dissolution and softening of the host tissue, so that it readily conformed to the shape of the metacercaria.

During the summer months, the metacercariae of *Apatemon g. minor* became encysted within 6 weeks after infection, whereas those of *Cercaria tetraglandis* did not encyst until 91 days after infection. During the winter months metacercariae of the latter species did not encyst until 139 days after infection. These facts, together with the differences noted in the degree of development between the metacercariae of *C. tetraglandis* obtained from a summer infection of 51 days duration and those from a 132 days winter infection experiment, suggest that temperature is an important factor in deciding the time required for the development and encystment of the metacercariae.

Szidat (1931) noted that the formation of the cyst wall occurred after 14 days in the metacercariae of *Apatemon g. gracilis*, Johnston *et al.* (1947, 1951) observed

the encystment of the metacercariae of *A. intermedius* in twenty-three days, whereas Willey and Rabinowitz (1938) found that encystment of the metacercariae of *A. g. burti* occurred between 30 and 40 days after infection. This variation may be a specific character or may be due to temperature effects but, unfortunately, no information is available on the temperature at which the development of the metacercariae of these species took place. Willey & Rabinowitz (1938) reported a 100% encystment of the metacercariae of *A. g. burti*, but, during the present life-cycle experiments, unencysted forms were found more than 200 days after infection and it is extremely doubtful whether these forms would have completed their development. It would not in fact be expected that a 100% encystment would occur, because not only is there a variation in viability and activity between individual cercariae, but there may also be the selection of an unfavourable site within the leech host. The well-developed musculature of the posterior sucker of the host appears to delay the penetration of the cercariae and it may similarly be responsible for a retardation in the rate of development of the metacercariae, as encysted forms have only rarely been observed within the posterior sucker.

The relatively long time required for the encystment of the metacercariae of *Cercaria tetraglandis* may indicate that *Erpobdella testacea* is not the most favourable host, and perhaps the Rhynchobdellids, in which *C. tetraglandis* is known to develop, may prove to be more suitable.

As has been previously noted, the leech host can withstand a very heavy infection with tetracotyle metacercariae and still be capable of reproduction. Following exposure to infection, the surface tissues of the leech may show local vesicular enlargements and superficial haemorrhages, which presumably result from the penetration of the cercariae. If death does not follow exposure, these vesicular swellings and haemorrhages are soon lost and the infection can only be observed by microscopical observations. Superficially situated metacercariae are often visible to the naked eye as whitish spots.

THE DEVELOPMENT OF THE METACERCARIAE

A. **Cercaria tetraglandis,** Iles (1959)

(1) *Host specificity*

(*a*) *First intermediate host*

Although several species of *Planorbis* occur in Roath Park Lake, Cardiff, *Cercaria tetraglandis*, Iles, has been found only in *P. corneus* and none of the other pulmonate or prosobranch gastropods also found in this locality has been observed to harbour this species. This suggests that the miracidium has, at least in nature, a rigid host specificity.

(*b*) *Second intermediate host*

Cercaria tetraglandis will penetrate into leeches of the genera *Erpobdella*, *Glossiphonia* and *Protoclepsis* as well as into the aquatic oligochaetes, *Tubifex* spp. It is unlikely that development would be completed within the latter, since observations made on the development of the metacercariae within the leeches suggest that

encystment does not occur rapidly, whereas the life of the oligochaetes is presumably short. It is possible that a secondary route of infection may exist, if the cercariae are capable of withstanding the digestive secretions of the leech, because these oligochaetes are eaten by several species of leeches.

(2) *Development*

The following observations have been made on the developmental stages of the metacercariae of this species. Some measurements of metacercariae at different ages are given in Table 2. In all these experiments it was obvious that the development of the metacercariae, resulting from a single infection with cercariae and developing in the same host, was not simultaneous.

Table 2. *Measurements (mm.) of metacercariae of different ages*

Cercaria tetraglandis

Age ...	6 days (living)		51 days (living)		132 days (living)	
	Range	Average	Range	Average	Range	Average
B.L.	0·13–0·20	0·167	0·18–0·43	0·308	0·17–0·57	0·319
B. (at V.S.)	0·04–0·09	0·070	0·16–0·26	0·210	0·06–0·30	0·142
(Post.)	—	—	0·05–0·15	0·110	—	—
O.S.	0·02–0·04 ×	0·030 ×	0·02–0·04 ×	0·035 ×	0·02–0·03 ×	0·024 ×
	0·02–0·03	0·028	0·03	0·030	0·02–0·03	0·024
V.S.	0·03–0·04 ×	0·034 ×	0·035–0·04 ×	0·038 ×	0·03 ×	0·03 ×
	0·03–0·04	0·034	0·040	0·040	0·03	0·03
Ad. org.	—	—	—	—	0·10 ×	0·10 ×
	—	—	—	—	0·10	0·10

Age ...	91 days (living, encysted)		153 days (living, encysted)	
	Range	Average	Range	Average
O.L.	0·40–0·48	0·447	0·42–0·62	0·487
O.B.	0·22–0·40	0·327	0·34–0·52	0·450
I.L.	—	—	—	—
I.B.	—	—	—	—
O.S.	0·05–0·07 ×	0·058 ×	0·05–0·06 ×	0·057 ×
	0·05–0·08	0·061	0·05–0·06	0·055
V.S.	0·05–0·07 ×	0·063 ×	0·060 ×	0·060 ×
	0·05–0·08	0·070	0·060	0·060
Ad. org.	0·07–0·09 ×	0·08 ×	0·10–0·24 ×	0·128 ×
	0·09–0·12	0 11	0·10–0·25	0·130

One to two days. Infected 20 September 1956. Dissected 21 and 22 September 1956.

Cercarial bodies examined 17 and 31 hr. after penetration were very active and little different from the cercariae, except that the penetration gland cells were no longer readily visible, although their ducts were still conspicuous. The presence of large refractile cells obscured the morphology. Szidat (1924, 1931) and Komiya (1938) observed similar cells in the developmental stages of other metacercariae. The entire nature of the cytoplasm had changed from a uniform finely granular structure to a refractile coarsely granular one.

Six days. Infected 4 December 1956. Dissected 10 December 1956.

The bases of the penetration gland cell ducts persisted in the proximal part of the oral sucker, but the remainder of the penetration gland cell system had disappeared. The spination was identical with that of the cercaria. The body had a greenish tinge and was opaque, but those portions of the excretory system that were observed indicated that the number and position of the flame cells in it was identical with that in the cercaria. The excretory bladder was still trilobed.

Fourteen days. Infected 28 November 1956. Dissected 12 December 1956.

The cercarial form was still maintained, but the body contents were very refractile and all trace of the penetration gland cell system had disappeared. No flame cells were visible, but the bladder remained trilobed. Three rows of spines were present on the anterior tip of the body, but no spines were seen on the ventral sucker or body margins. These metacercariae were slightly larger than those obtained 6 days after infection.

Twenty-one days. Infected 28 November 1956. Dissected 19 December 1956.

The metacercariae were less active than those obtained 4, 6 and 14 days after infection. No spines were visible. The pharynx was conspicuous, but the gut caeca were only made out with difficulty. The bladder was still trilobed. There had been a progressive increase in the size of the metacercariae.

Fifty-one days. Infected 3 July 1956. Dissected 23 August 1956.

The majority of the metacercariae had the characteristic pyriform shape of the tetracotyle, although some were little different from the cercariae in size and morphology. The tissues were very refractile and numerous greenish granules were distributed between and within the large vacuolate cells. The pharynx and gut were inconspicuous, but the oral and ventral suckers were still distinct. Only six pairs of flame cells were seen, but undoubtedly more were present but were hidden by the refractile granular nature of the body. Neither the adhesive organ nor lateral sucking cups were yet developed.

Ninety-one days. Infected 6 July 1956. Dissected 1 October 1956.

Most of these metacercariae were encysted and the cyst wall consisted of several concentric layers, although again some were at a much earlier stage of development. The lateral cups and adhesive organ were readily visible and the oral and ventral suckers were still conspicuous and relatively large. Twenty-one pairs of flame cells were observed, but probably more were present but hidden, as were the connexions of the observable flame cells with the primary excretory system. The flame cells appeared to be grouped in threes. The excretory pore was subterminal and dorsally situated and the large excretory spaces of the reserve bladder system were evident.

One hundred and thirty-two days. Infected 26 October 1956. Dissected 7 March 1956.

Most of these metacercariae were not encysted, but possessed an elongate oval form without a distinct hind-body and some were still very similar to the cercariae in size and structure. The lateral sucking cups of the former were indistinct and the adhesive organ was clearly developed in only one specimen. They were smaller than those metacercariae obtained 51 days after infection and at a much

earlier stage of development than those obtained 91 days after infection. This apparent anomaly can be explained when the dates of infection are compared. Those examined 51 and 91 days after infection were from experiments conducted during the summer months and had developed at a higher temperature than this infection which had extended through the winter months.

One hundred and thirty-nine days. Infected 19 October 1956. Dissected 7 March 1957.

The majority of these were encysted and within the same range of size as the 91-day-old encysted metacercariae. The outer cyst wall varied in thickness between 0·05 and 0·13 mm. A few were observed which possessed only the thin inner cyst wall; some were unencysted, but had well-developed and conspicuous lateral sucking cups and adhesive organ; and others had undergone very little development and closely resembled the cercarial stage.

One hundred and fifty-three days. Infected 20 September 1956. Dissected 20 February 1957.

Both unencysted and encysted forms were present in this infection. Some of the unencysted metacercariae had changed very little from the cercarial phase; others were larger than the encysted forms and possessed the lateral lappets and adhesive organ characteristic of the tetracotyle. The bilobed nature of the excretory bladder was evident and the large excretory spaces of the reserve bladder system extended throughout the fore-body. Ramifications of the primary excretory system were also traced, but the capillaries of the flame cells were not visible. The pharynx was small and placed close to the oral sucker. The oesophagus bifurcated anteriorly and the gut caeca terminated at approximately one-third of the length of the adhesive organ. The encysted forms were slightly larger than those obtained from the infection of 91 and 139 days duration (Table 2). The oral and ventral suckers and the adhesive organ also were larger than those of the metacercariae of the latter infections. The lateral lappets were set 0·14–0·18 mm. apart and had a diameter of 0·03 mm.

Attempts to elucidate completely the structure of the primary excretory and reserve bladder systems were unsuccessful because of the opaque granular nature of the body. The variation with age in the size of the encysted metacercariae indicates that measurements alone are not a satisfactory basis for specific determination.

B. Cercaria Apatemon gracilis minor

Komiya (1938) first described *C. A. g. minor* as *C. hamburgensis* and followed the development of the excretory system from the germ-ball stage to that of the cercaria. He also noted that this species penetrated into *Erpobdella* spp., but he did not follow its development into a tetracotyle metacercaria within this host.

(1) *Host specificity*

(*a*) *First intermediate host*

This cercaria was found developing only in *Lymnaea pereger* from Roath Park Lake, Cardiff. Several other species of pulmonate and prosobranchiate molluscs

occur in the same locality, such as *Lymnaea*, *Planorbis* and *Bithynia* spp., but they have never been found to harbour this species. Komiya, however, has recorded it from *Lymnaea ovata*, Drap. and *L. palustris*, Müll, in Germany.

(*b*) *Second intermediate host*

Leeches belonging to the genera *Erpobdella*, *Glossiphonia* and *Protoclepsis* were introduced into vessels containing recently emerged cercariae, and these were seen to attach to, and penetrate into, all the different species. The cercariae never attempted to penetrate into the first intermediate host or into *Tubifex* spp.

Erpobdella testacea was the species used in the life-cycle experiments, because of its relative abundance and availability.

(2) *The distribution and development of the metacercaria*

Cercaria Apatemon g. minor is very similar in its morphology and the course of its development to *C. A. intermedius*, *C. A. g. burti* and *C. A. g. gracilis*. The life cycles of the other species of cercariae closely related to *C. A. g. minor* are not known, but it is probable that they, also, would penetrate into and develop within fresh-water leeches. These are *C. helvetica* XXXI (Dubois, 1929), *C. pygocytophora* (Brown, 1931), *C. pseudoburti* (Rankin, 1937), *C. okobojensis* (Brooks, 1943), *C. icnusae* (Giovannola, 1937) and *C. wansoni* (Fain, 1953). It is interesting to note that with the exception of *C. A. g. gracilis*, which develops within the prosobranchiate snail *Bithynia tentaculata*, all these species develop within pulmonate gastropods.

In the three species in which the development is known the metacercariae localize within the blood vascular system of the leech host. Szidat (1931) gave details of the location of the metacercariae of *A. g. gracilis* within this system. He observed the larvae in the ventral blood vessel and in the contractile ampullae of the vascular system. Sections were cut of experimentally infected specimens of *Erpobdella testacea* to determine the distribution of the metacercariae of *A. g. minor* within the host tissues. It was found that they did not localize within the blood vessels, but were distributed throughout the body tissues, often in close proximity to, though not within, the blood vessels (Pl. I), which are readily distinguished by their contents and by a surrounding layer of botryoidal tissue. As in the experiments in which *C. tetraglandis* was used, metacercariae resulting from a single infection of a leech were obtained at markedly different stages of development. Encystment occurred within 6 weeks after the infection of the leech. The average size of ten living encysted metacercariae at 234 days was $0{\cdot}378 \times 0{\cdot}307$ mm. Fixed cysts of the same age measured $0{\cdot}355 \times 0{\cdot}265$ mm. They are within the same size-range as the encysted metacercariae of *A. g. gracilis* and *A. g. burti*, but are larger than those of *A. intermedius* (Table 1).

(3) *Final host*

Day-old Khaki Campbell ducklings were force-fed with experimentally infected leeches and adult trematodes were obtained in the proximal portion of the small intestine. Adults were also obtained when ducklings 13 days old were force-fed

with infective material, a fact which indicates that an age immunity did not exist at that stage. Laboratory-bred rats were also force-fed with metacercariae, but neither excystation nor development was observed. Encysted metacercariae were recovered from the rectum of these rats and appeared to have been partly digested. The larvae within the cysts did not exhibit any activity and seemed to have been killed during the passage through the gut.

(4) *Excystation and the morphology of the excysted metacercariae*

Excysted metacercariae were not observed in the crop, stomach, duodenal loop or the hind-gut, but only in the small intestine. It is probable that excystation is initiated by the intestinal secretions, although previous exposure to gastric juices may be necessary. The failure of the metacercariae to excyst within the intestine of a rat suggests that the conditions required are peculiar to the intestine of birds. Possibly the higher body temperature or the nature of the enzymic secretions of the intestinal wall are involved. Excystation usually occurred within the 24 hr. following infection. The majority of metacercariae obtained after this time were completely freed from the cyst, but a few were still enclosed within the thin, inner cyst wall.

Excysted metacercariae obtained 24 and 40 hr. after infection had a large fore-body with conspicuous lateral sucking cups and an adhesive organ in addition to the oral and ventral suckers and a relatively small, poorly-delimited, hind-body which was almost completely filled by the bilobed excretory bladder. The rudiments of the reproductive organs situated in the hind-body were poorly developed and were clearly visible only in fixed and stained specimens. The ventral sucker was slightly larger than the oral sucker. The subterminal mouth was followed by a short prepharynx and a small, oval pharynx. The short oesophagus bifurcated some distance anterior to the acetabulum and the caeca extended to a point mid-way along the length of the adhesive organ. Numerous flame cells were seen, but the opacity of the body, resulting from the presence of refractile granules, prevented the exact number and arrangement from being determined. It is doubtful if the flame cell formula could be of any systematic value, because observation of it is difficult and possibly inaccurate. The reserve bladder system consisted of a series of large spaces containing a refractile fluid, through which a relatively rapid circulation occurred during the rhythmic contractions and expansions of the body. The excysted forms remained alive for 12–24 hr. in mammalian Ringer solution. They probed the environment with the adhesive organ and lateral sucking lappets and sudden pronounced contractions of the bladder caused excretory granules to be expelled through the dorsal, subterminal excretory pore. Measurements of both living and fixed specimens obtained 40 hr. after infection are given in Table 3.

(5) *Infectivity*

It was not possible to determine the exact number of metacercariae administered in the infection experiments. A suspension of a known number of metacercariae would not give this information, because the number lost by regurgitation could

not be estimated and the number of encysted metacercariae within the heavily infected leeches which were force-fed could not be accurately determined. The youngest metacercariae used in the infections were 104 days old and the oldest 234 days old. Although every infection yielded adult trematodes the relatively

Table 3. *Measurements (mm.) of adults of different ages*

Age ...	40 hours (living)		4 days (living)		10 days (living)		20 days (living)	
	Range	Average	Range	Average	Range	Average	Range	Average
F.B.L.	0·21–0·35	0·270	0·30–0·40	0·340	0·36–0·67	0·520	0·54–0·84	0·662
F.B.B.	0·21–0·29	0·250	0·29–0·42	0·320	0·32–0·46	0·427	0·39–0·64	0·514
H.B.L.	0·09–0·15	0·118	0·30–0·52	0·410	0·94–1·27	1·111	1·11–1·68	1·430
H.B.B.	0·06–0·16	0·134	0·26–0·37	0·310	0·34–0·48	0·427	0·48–0·64	0·578
O.S.	0·05–0·07×	0·063×	0·07–0·11×	0·090×	0·08–0·09×	0·089×	0·10–0·12×	0·107×
	0·05–0·06	0·059	0·08–0·12	0·089	0·08–0·10	0·094	0·10–0·12	0·108
V.S.	0·06–0·09×	0·07×	0·07–0·12×	0·09×	0·06–0·15×	0·122×	0·13–0·16×	0·154×
	0·05–0·10	0·077	0·10–0·13	0·12	0·10–0·16	0·140	0·14–0·20	0·174
Ad. Org.	0·11–0·25×	0·164×	0·16–0·30×	0·21×	0·22–0·45×	0·311×	0·24–0·40×	0·303×
	0·07–0·18	0·133	0·12–0·28	0·17	0·21–0·40	0·282	0·21–0·55	0·436
T. ant.	—	—	0·06–0·21×	0·11×	0·12–0·25×	0·189×	0·21–0·37×	0·309×
	—	—	0·14–0·20	0·16	0·10–0·20	0·159	0·21–0·33	0·259
T. post.	—	—	0·06–0·12×	0·08×	0·12–0·24×	0·177×	0·25–0·42	0·345×
	—	—	0·16–0·26	0·16	0·12–0·24	0·159	0·21–0·32	0·249
Ovary	—	—	—	—	0·10–0·16×	0·113×	0·11–0·24×	0·158×
	—	—	—	—	0·08–0·15	0·129	0·13–0·23	0·179
Eggs	—	—	—	—	0·09–0·11×	0·108×	0·10–0·11×	0·109×
	—	—	—	—	0·05–0·07	0·062	0·06	0·06

	Fixed (6 specimens)		Fixed		Fixed		Fixed*	
	Range	Average	Range	Average	Range	Average	Range	Average
F.B.L.	0·18–0·22	0·20	0·21–0·30	0·26	0·31–0·39	0·342	0·25–0·36	0·321
F.B.B.	0·11–0·19	0·16	0·22–0·27	0·24	0·27–0·45	0·348	0·26–0·39	0 304
H.B.L.	0·03–0·08	0·06	0·30–0·39	0·33	0·60–0·83	0·717	0·53–0·76	0·606
H.B.B.	0·06–0·09	0·08	0·16–0·22	0·19	0·20–0·32	0·250	0·21–0·32	0·246
O.S.	0·03–0·05×	0·045×	0·06–0·08×	0·07×	0·07–0·09×	0·083×	0·07–0·09×	0·083×
	0·04–0·05	0·043	0·06–0·08	0·07	0·06–0·10	0·086	0·07–0·10	0·085
V.S.	0·04–0·05×	0·045×	0·07–0·10×	0·08×	0·06–0·12×	0·098×	0·08–0·10×	0·088×
	0·04–0·05	0·045	0·07–0·12	0·09	0·09–0·13	0·107	0·09–0·12	0·102
Ad. org.	0·09–0·21×	0·14×	0·11–0·20×	0·14×	0·13–0·32×	0·194×	0·10–0·16×	0·131×
	0·05–0·12	0·10	0·12–0·22	0·19	0·16–0·36	0·262	0·21–0·30	0·245
T. ant.	—	—	0·07–0·11×	0·09×	0·15×	0·15×	0·14×	0·14×
	—	—	0·09–0·12	0·11	0·10†	0·10	0·11†	0·11
T. post.	—	—	0·06–0·08×	0·07×	0·11–0·15×‡	0·091×	0·15×	0·15×
	—	—	0·11–0·12	0·12	0·07–0·11	0·090	0·10†	0·10
Ovary	—	—	—	—	0·10×	0·10×	0·08×	0·08×
	—	—	—	—	0·15†	0·15	0·09†	0·09
Eggs	—	—	0·09×	0·09×	0·09–0·10×	0·095×	0·09–0·10×	0·094×
	—	—	0·005†	0·005	0·05	0·05	0·05	0·05

* These specimens contracted excessively on fixation. † 1 specimen. ‡ 2 specimens.

low numbers (from 1 to 56) recovered in all the infections indicated a low percentage infectivity of the metacercariae. Stunkard *et al.* (1941) suggested that a period of development within the cyst was necessary, because metacercariae of *A. g. burti* from a 38-day infection were infective, whereas those from a 34-day infection were not.

(6) *Adult*

The majority of adult trematodes were found in the first 8 in. of the small intestine following the duodenal loop, but a few were found in the mid-gut region. Adults were obtained 4, 6, 10, 14 and 20 days after infection and recently excysted metacercariae were recovered 24 and 40 hr. after the administration of infective material to the ducklings. Some measurements are given in Table 3. The greatest development occurred between the second and fourth days with the extension of the hind-body and the growth and rapid differentiation of the reproductive organs. Between the fourth and sixth days there was an increase in size of the fore- and hind-bodies and between the tenth and twentieth days there was a further but less-marked increase in size (Table 3), shown by measurements of living specimens. The marked difference between the measurements of living and fixed specimens is the result of the strong contraction which occurred on fixation and of shrinkage during the staining and mounting procedures. The majority of descriptions of adult trematodes available have been based on preserved specimens and the fixative and number of specimens examined are usually not given, so that it is impossible to obtain comparative data on measurements. The age of trematodes obtained from natural infections is not known and, because size varies with age, at least in this species, it could result in confusion when specific diagnoses are based on measurements.

In adults obtained 4 days after infection the reproductive organs were differentiated, but of the thirty-five specimens recovered one, only, contained a single egg and that was in the proximal limb of the uterus. Several of the trematodes obtained 6 days after infection contained one or two eggs in the uterus, but no eggs were found within the gut of the duckling or the faeces suggesting that, although the egg production had begun, deposition of ova had not. This would undoubtedly soon follow, because some of the eggs were in the distal portion of the uterus, were deeply pigmented and would obviously soon be shed. Numerous eggs were found within the intestine and faeces of ducks 10, 14 and 20 days after infection. It is an interesting fact that the adults with the largest number of eggs in the uterus were obtained from the infection of 20 days duration.

The genus *Apatemon* is characterized by the presence of a genital cone, which is traversed by a hermaphrodite duct, formed by the union of the ductus ejaculatorius and the uterus. In the majority of adults recovered from the experimentally infected ducklings active sperm was aggregated at the tip of the genital cone and on several occasions eggs were observed being extruded from this greatly distensible canal.

Apatemon gracilis minor was first described by Yamaguti (1933) from Japan and has more recently been recorded by Dubois & Rausch (1948) from America and by Baer (1956) from Greenland. The specimens obtained in these life-cycle experiments differed from those described by Yamaguti only in the greater variation in the size of the eggs. This may be because I measured both non-pigmented and pigmented eggs, whereas Yamaguti may have measured only those ready for deposition. The measurements of the fixed specimens fall within the range given by Yamaguti.

Apatemon gracilis burti, Dubois also falls within the same size range, but it may be distinguished from *A. g. minor* by the contiguous position of the testes and their more anterior position and by the presence of slender intestinal caeca which Stunkard *et al.* (1941) suggested were non-functional. The caeca of *A. g. minor* are large and conspicuous, particularly in living material and are obviously functional. Only one specimen of *A. g. minor* with contiguous testes was seen by me during these infection experiments. Marked differences exist in the biology of the adults of these two species. In the life-cycle studies on *A. g. burti* (Stunkard *et al.*) eggs were recovered from the faeces of the host 4 days after the administration of the infective metacercariae. The number increased until the eighth day and then decreased until the thirteenth day, when adults were no longer found in the intestine. The longevity of *A. g. burti* is therefore of the order of 12 days and much less than that of *A. g. minor*, which 20 days after infection was still producing large numbers of eggs. This may be correlated with the fact that there is a more rapid attainment of maturity and egg production in the former species than in *A. g. minor*. *A. g. gracilis* differs markedly from *A. g. minor* in size and the morphology and biology of the related larval stages.

It is apparent that in determining the species of the adult both morphological and biological characters of the cercariae and metacercariae should be considered. The subspecies of *A. gracilis* cannot be readily separated on morphological characters alone, but they are easily distinguished when the biology of the adult and larval stages is taken into account.

DISCUSSION

During this study several important facts have emerged with reference to the development of the metacercariae and the importance of biological characters in the classification of the adult and larval stages of the digenetic trematodes. Observations on the development of the metacercaria of *C. tetraglandis* indicate that the cercarial characters are gradually lost or altered and are superseded by those of the metacercaria and that, in addition to these morphological changes, physiological adaptations must also occur to allow the metacercaria to adjust to the conditions within the leech host, which differ markedly from those under which the free-living cercaria exists. It was noted that the development of the metacercariae resulting from a single infection and within the same host is not simultaneous, the same leech host harbouring forms which had encysted and others little different from the cercaria in their morphology. It was also apparent that the development and encystment of the metacercariae were influenced by the temperature at which they occurred. Infection experiments extending through the winter months and those completed during the summer months showed a marked difference in the time at which encystment occurred.

The slight differences in the morphology of the adult stages of the subspecies of *A. gracilis* assume greater significance when they are found to be correlated with differences in the biology of these adults and with the morphology and biology of the related larval forms. In the case of the adults, in addition to data on the

morphology and biology of the adult, which would include, if possible, information on the longevity, the time required to reach maturity and at which egg deposition begins, it is important, also, to know the age of the specimens, the number examined and measured and whether they were living or fixed and the fixative used, because all these factors affect to an appreciable extent the size and proportions of the specimen. Similarly any attempt to differentiate tetracotyle metacercariae on the basis of measurements would be of little value in the absence of data on their variation, age, their state, living or fixed, the fixative used and the number of specimens measured. Any differences recorded when all these factors have been considered assume significance in combination with differences in the biology and, particularly, the tissue and host specificity. The excretory system is of little value in the specific determination of trematode metacercariae or adults because of its complexity and the difficulty of observation, although the gross structure of the reserve bladder system in the metacercariae (Hughes, 1928; Van Cleave & Mueller, 1934 and Komiya, 1938) is of value in indicating the type of metacercaria. The known metacercariae of the subspecies of *A. gracilis* are all tetracotyliform larvae, with the exception of that of *A. g. burti*, which is intermediate in structure between the tetracotyle and diplostomulum types and possesses several unique features (Stunkard *et al.* 1941).

The flame cell and the penetration gland cell systems are of use in the specific diagnosis of cercariae, although closely related forms often have an identical flame cell formula and penetration gland cell system and differ only in details of spination, size or behaviour. The exact systematic significance of these latter characters can only be assessed when more knowledge is available on the life cycles of closely related species.

SUMMARY

1. Observations have been made on the life cycles of *Cercaria paracauda*, Iles, *C. tetraglandis*, Iles, and *C. Apatemon gracilis minor*, Yamaguti. The development of the tetracotyle metacercaria of *C. tetraglandis* has been followed and also that of the metacercaria and adult of *A. g. minor*. The pathology and distribution of the metacercariae of the last two species within the leech, *Erpobdella testacea* (Sav.) has been studied.

2. It was noted that development of the metacercariae within the same host and resulting from the same infection was not simultaneous and that it was improbable that a 100% encystment of the metacercariae would occur. Temperature was found to have a marked effect on the length of time required for encystment.

3. Data are given on the infectivity and excystation of the metacercariae of *A. g. minor* and on the biology and morphology of the adult of this subspecies.

4. Stress is placed on the importance, in the systematics of the digenetic trematodes, of considering both the morphological and biological characters of the adult and those of the related larval stages.

I wish to acknowledge the assistance of Dr G. Dubois in the identification of the adult specimens of *Apatemon gracilis minor* Yam. obtained by experimental infection. I am grateful to Mr S. Nield of Windermere for the collection of numerous specimens of fresh-water leeches.

REFERENCES

BAER, J. (1956). Parasitic helminths collected in West Greenland. *Meddelsomer om Grønland*, **124**, 1–55.

BROOKS, F. G. (1943). Larval trematodes of Northwest Iowa. II. Four new strigeids. *J. Parasit.* **29**, 340–7.

BROWN, F. J. (1931). Some freshwater larval trematodes from Cheshire. *Parasitology*, **23**, 88–98.

DUBOIS, G. (1929). Les cercaires de la région de Neuchâtel. *Bull. Soc. neuchâtel. Sci. nat.* **54**, 3–177.

DUBOIS, G. & RAUSCH, R. (1948). Seconde contribution à l'étude des Strigeides (Trematoda) Nord-Americains. *Bull. Soc. neuchâtel. Sci. nat.* **71**, 29–61.

ERASMUS, D. A. (1958). Studies on the morphology, biology and development of a strigeid cercaria (*Cercaria X* Baylis, 1930). *Parasitology*, **48**, 312–34.

FAIN, A. (1953). Contribution à l'étude des formes larvaires des trématodes au Congo belge et spécialement de la larva de *Schistosoma mansoni*. *Inst. Roy. Colon. Belge. Sect. Sci. Nat. Méd.* **22**, 1–312.

GIOVANNOLA, A. (1937). Su due nuove cercariae studiate in Sardegna. *Riv. Parassit.* **1**, 31–7.

HARPER, W. F. (1931). On the structure and life history of British fresh water furcocercariae. *Parasitology*, **23**, 310–24.

HUGHES, R. C. (1928). Studies on the trematode family Strigeidae (Holostomidae) XIII. Three new species of Tetracotyle. *Trans. Amer. Micr. Soc.* **47**, 414–33.

ILES, C. (1959). The larval trematodes of certain fresh-water molluscs. I. The furcocercariae. *Parasitology*, **49**, 478–505.

JOHNSTON, T. H. & ANGEL, L. M. (1951). The morphology and life cycle of the trematode *Apatemon intermedius* from the black swan. *Trans Roy. Soc. S. Aust.* **74**, 66–78.

JOHNSTON, T. H. & BECKWITH, A. C. (1947). Larval trematodes from Australian fresh-water molluscs. Part XI. *Rec. S. Aust. Mus.* **8**, 563–83.

KOMIYA, Y. (1938). Die Entwicklung des Excretionssystems einiger Trematodenlarven aus Alster und Elbe nebst Bemerkungen über ihren Entwicklungszyklus. *Z. Parasitenk.* **10**, 340–85.

LUTZ, A. (1921). Zur Kenntnis des Entwicklungszyklus der Holostomiden. Vorläufige Mitteilung. *Zbl. Bakt.* Abt. 1, Orig., **86**, 124–9.

RANKIN, J. S. (1937). *C. pseudoburti*, a strigeid cercaria from Western Massachusetts. *J. Parasit.* **25**, 87–91.

STUNKARD, H. W., WILLEY, C. H. & RABINOWITZ, Y. (1941). *C. burti* Miller 1923, a larval stage of *Apatemon gracilis* (Rudolphi, 1819) Szidat, 1928. *Trans. Amer. Micr. Soc.* **60**, 485–97.

SZIDAT, L. (1924). Beiträge zur Entwicklungsgeschichte der Holostomiden. II. *Zool. Anz.* **61**, 249–66.

SZIDAT, L. (1929). Beiträge zur Entwicklungsgeschichte der Holostomiden. III. Ueber zwei Tetracotylen aus Hirudineen und ihre Weiterentwicklung in Enten zu *Cotylurus cornutus* und *Apatemon gracilis* Rud. *Zool. Anz.* **86**, 133–49.

SZIDAT, L. (1931). Beiträge zur Entwicklungsgeschichte der Holostomiden. IV. Die Cercariae des Enten parasiten *Apatemon* (Strigea) *gracilis* Rud. und ihre Entwicklung im Blutgefässsystem des Zwischenwirtes (*Herpobdella atomaria* Car.) *Z. Parasitenk.* **3** (2), 160–72.

VAN CLEAVE, H. J. & MUELLER, J. F. (1934). Parasites of Oneida Lake fishes. Part III. A biological and ecological survey of the worm parasites. *Roosevelt Wild Life Ann.* **3**, (3, 4), 161–334.

WILLEY, C. H. & RABINOWITZ, Y. (1938). The development of *C. burti* Miller, 1923, in leeches and ducks. *J. Parasit.* **24**, Suppl. 30–1.

WISNIEWSKI, L. W. (1935). *Cercaria dubia*, sp.n. und deren weitere Entwicklung in *Herpobdella atomaria* *Bull. int. Acad. Cracovie* (*Acad. pol. Sci.*) B, (II), (1–2), 19–35.
YAMAGUTI, S. (1933). Studies on the helminth fauna of Japan. Part I. Trematodes of birds, reptiles and mammals. *Jap. J. Zool.* **5**, 1–134.

Abbreviations

Ad. org.	adhesive organ	I.L.	length excluding cyst wall
B.	breadth	L.	length
B.B.	body breadth	O.B.	breadth including cyst wall
B.L.	body length	O.L.	length including cyst wall
F.B.B.	fore-body breadth	O.S.	oral sucker
F.B.L.	fore-body length	Post.	posterior
H.B.B.	hind-body breadth	T.ant.	anterior testis
H.B.L.	hind-body length	T.post	posterior testis
I.B.	breadth excluding cyst wall	V.S.	ventral sucker.

EXPLANATION OF PLATE

Fig. 1. L.S. of *Erpobdella testacea* showing the distribution of the metacercaria of *Apatemon gracilis minor*.

Fig. 2. L.S. of *Erpobdella testacea*. Metacercaria of *Apatemon gracilis minor* showing attachment of cyst wall to host tissue.

Fig. 1

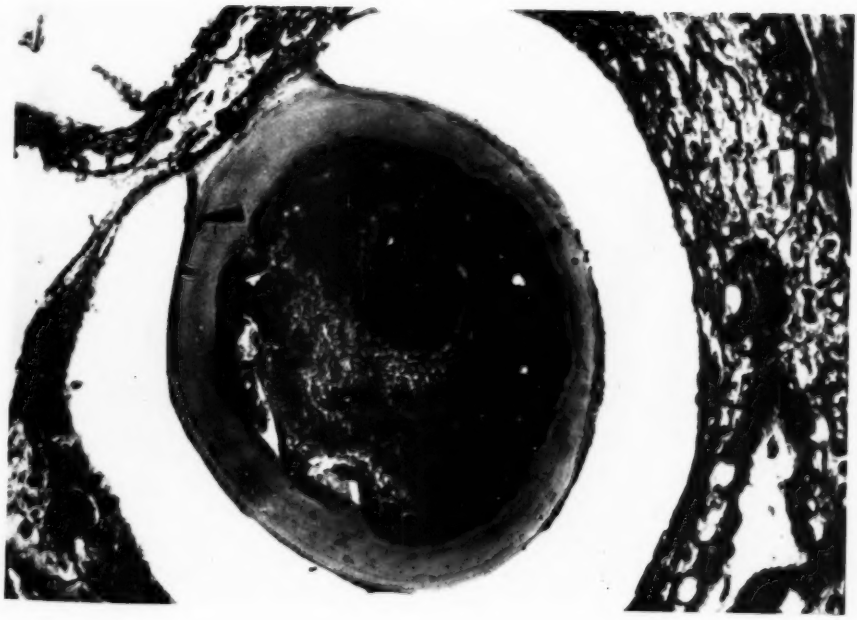


Fig. 2

CHRISTINE ILES

(*Facing p.* 418)

Parasitology (1960), **50**, 419–423
With 2 text-figures
Printed in Great Britain

The nature of the cytoplasm-modifying antibodies

By C. KULASIRI*

Department of Parasitology, London School of Hygiene and Tropical Medicine, London, England

(*Received* 6 *January* 1960)

INTRODUCTION

Changes in the serum protein pattern of mice intraperitoneally infected with *Toxoplasma* were observed by Nagaoka (1957). Twelve hours after the inoculation, he observed a slight increase in the total serum proteins followed by a decrease. The total proteins showed a marked increase after 72 hr. However, the albumin fraction steadily decreased following inoculation and after 96 hr. it became less than half the normal amount. The globulin fraction increased to more than twice the normal amount, each of the fractions α_1-, α_2-, β- and γ-globulins showing an increase. At a later stage it was seen that the β- and γ-globulins were specially increased. Wildführ, Naumann & Wilde (1958) observed, in rats undergoing active immunization with *Toxoplasma*, an increase in the total proteins of the majority of animals followed by a fall. When the elution technique was used in conjunction with paper electrophoresis, only small variations were seen among α_1-, α_2- and β-globulins and these were regarded as being insignificant. Generally the albumin fraction fell during immunization and the γ-globulin fraction increased.

Goldman (1957*a*) found no difference in the cytoplasm-modifying antibody titre (dye-test titre) of the original human serum and its globulin portion separated by half saturation with ammonium sulphate. He also observed (1957*b*) that the globulin fraction of pooled sera from experimentally infected rabbits had a cytoplasm-modifying antibody titre of 1 : 16,000. Although the work of Nagaoka (1957) and Wildführ *et al.* (1958) showed an increase in the γ-globulin fraction and that of Goldman (1957*a*, *b*) showed that the cytoplasm-modifying antibodies are associated with globulins, no attempt has been so far made to establish which fraction of the globulins contains the cytoplasm-modifying antibodies. The following study has been undertaken for this purpose.

MATERIALS AND METHODS

Sera from rabbits infected with the avirulent 'RB 25' strain of *Toxoplasma gondii* were used. The method adopted for separating the various fractions of proteins was the alcohol precipitation at low ionic strengths, low temperatures and controlled pH as developed by Cohn, Luetscher, Oncley, Armstrong & Davis (1940). This method was suitably altered to meet the changed conditions under which the precipitation was conducted. The protein separation was carried out in small

* Present address: Medical Research Institute, Colombo 8, Ceylon.

quantities. All salt solutions used had an ionic strength of 0·01 M at room temperature, except where otherwise stated. The alcohol added at various stages was not included in the determination of the ionic strength. All pH measurements were made at room temperature (22° C.) using a Cambridge pH meter. Dialysis was carried out using cellophane tubing in a room maintained at 2–3° C. The centrifuging was done in a M.S.E. angle-head bench centrifuge placed in this cold room and used at full speed. The temperature after centrifuging for 15–20 min. was between 3 and 4° C.

Five millilitres of the rabbit serum were dialysed against a sodium acetate-acetic acid buffer of pH 6·2 for 48 hr., with changes of the buffer at intervals. At the end of this period some of the proteins were observed to have precipitated. The pH of the serum suspension, which was now round 6·8, was adjusted to 6·2 with sodium acetate-acetic acid buffer of pH 4·3. The volume of the protein suspension was made up to 10 ml. with sodium acetate-acetic acid buffer of pH 6·2. This suspension was then allowed to cool and 3·3 ml. of precooled ethanol were added with stirring. The mixture, the pH of which was 6·4, was left in the cold room overnight. The precipitate was separated by centrifuging for 15–20 min. and the centrifuge tube was rinsed with cooled acetate-acetic acid buffer of pH 6·2 containing the same percentage of alcohol (30 %). The supernatant contained most of the albumin and α-globulin (fraction S). This was dialysed against distilled water for 48 hr. and freeze-dried. The precipitate, which contained the γ-globulin, β-globulin, traces of α-globulin and albumin, was dissolved in 12·5 ml. of an acetate-acetic acid-carbonate buffer of pH 6·6 prepared by mixing 10 ml. of a sodium acetate-acetic acid buffer of pH 5·1 and 2·5 ml. of a sodium carbonate solution of pH 9·8. Some of the proteins however did not dissolve and the suspension was allowed to stand for $\frac{1}{2}$ hr. and the undissolved proteins were separated by centrifuging for 15–20 min. The centrifuge tube containing the precipitate was rinsed three or four times with the same buffer solution. The precipitate was dissolved in 5 ml. of isotonic phosphate-buffered saline at pH 7·6 (fraction A). To the supernatant of this centrifugation, 3·5 ml. of precooled ethanol were added with constant mixing. The alcohol concentration of this mixture was 21·9 % and the pH 7·3. The suspension was allowed to stand for an hour in the cold room and the precipitate separated by centrifuging for 15–20 min. The supernatant thus obtained (fraction B), which contained most of the β-globulin and traces of α-globulin and albumin, was dialysed against distilled water for 48 hr. and freeze-dried. The precipitate, mixed with 12·5 ml. of buffer of pH 6·6 prepared as above, is completely dissolved within 30 min. To reprecipitate the γ-globulin, 3·5 ml. of precooled ethanol were added and the mixture was allowed to stand in the cold room for 30 min. before the precipitate in it was separated by centrifuging for 15–20 min. The supernatant thus obtained (fraction B 1) was dialysed against distilled water for 48 hr. and freeze-dried. The centrifuge tube containing the precipitate was rinsed four to five times with cooled alcohol-buffer mixture of the same composition as the supernatant and the precipitate was dissolved in 5 ml. of isotonic phosphate-buffered saline. The freeze-dried fractions S, B and B 1 were each dissolved in 5 ml. of isotonic phosphate-buffered saline to bring them to the original volume.

The various fractions of proteins were analysed by paper electrophoresis for purity of fractions. The method adopted for this purpose was essentially that described by Flynn & de Mayo (1951). A Durrum type of cell was used with a barbiturate buffer of pH 8·6 and an ionic concentration of 0·01 M, with Whatman's 3M paper. Suitable lengths were cut from the roll and moistened in the buffer solution. The excess buffer was absorbed by means of filter paper. 0·1 ml. of the serum was applied as a band about an inch below the centre of the paper on the anode side. A d.c. electric current at 80 V. was allowed to flow through the paper for a period of 16 hr. During this period of time the bands were separated by about 16 cm., which was about the length of strips that could be accommodated in the scanner. The proteins were fixed to the paper by drying in an oven at about 100° C. for 15 min. The dried strips were stained for 10 min. in a dye solution prepared by

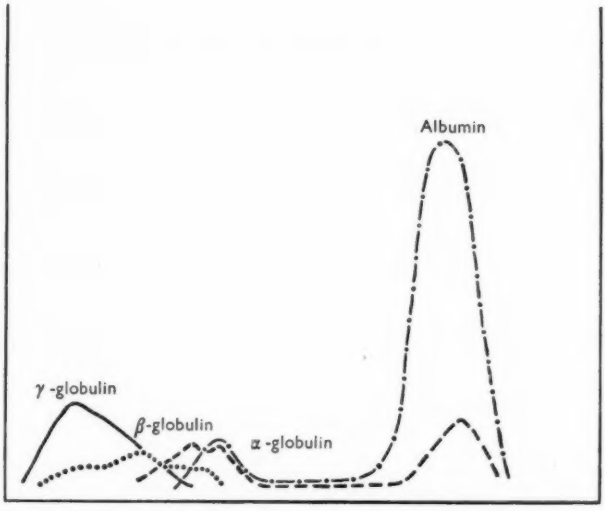


Fig. 1. Curves of the protein fractions obtained by alcohol precipitation ···, Fraction A; – – –, fraction B; —, fraction C; · — · — · fraction S.

saturating a mixture of equal volumes of methanol and 10% aqueous acetic acid with azocarmine B (C.I. no. 829; see Conn, 1953). The strips were then washed in three rapid changes of tap water to remove the excess dye solution and for 5 min. in methanol containing 10% acetic acid. The strips were then differentiated for a further 5 min. in 10% acetic acid in water and were then allowed to drip dry. They were trimmed into portions of suitable size for the scanner and made translucent by soaking in a solution containing equal parts of 1-bromonaphthalene and liquid paraffin of specific gravity 0·830–0·870. The excess of the 'clearing agent' was removed by blotting with filter paper. The reading was carried out using an EEL photoelectric 'Scanner' with a green filter. The dye tests were carried out on the various fractions as described previously (Kulasiri, in press).

RESULTS

Fig. 1 shows the results of the fractionation. The curves A, B, C and S correspond to the electrophoretic patterns obtained from the fractions A, B, C and S, respectively. Fig. 2 shows the protein pattern of the original serum. Fraction A (curve A) contains γ-globulin and some β-globulin. Fraction B (curve B) contains β-globulin, α-globulin and a little albumin. Fraction B 1 did not show any protein fractions at the quantity used. The whole of the fraction C (curve C) was γ-globulin. Fraction S (curve S) contains α-globulin and albumin. The normal serum had a dye-test titre of 1:256. The results of the dye tests on the fractions are shown in Table 1. Fractions A and C gave titres of 1:64 and the other fractions, titres of 1:4.

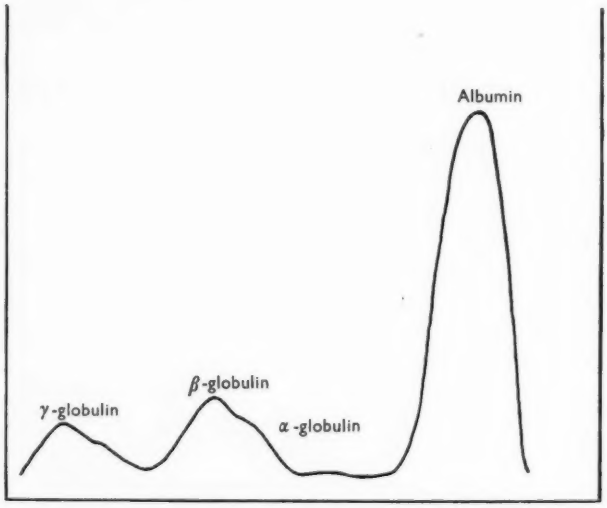


Fig. 2. Curve of the positive serum before alcohol precipitation.

Table 1. *Dye-test titres obtained in normal serum and serum fractions*

Fraction	Protein constituents	Dye-test titre
A	γ- and β-globulins	1:64
B	β-, α-globulins and albumin	1:4
B 1	No proteins detected electrophoretically	1:4
C	γ-globulin	1:64
S	α-globulin and albumin	1:4
Normal serum	All protein fractions	1:256

DISCUSSION

The γ-globulin of the serum was contained in the two fractions A and C. Of these, fraction C consisted wholly of γ-globulin, while fraction A contained a fair quantity of β-globulin associated with γ-globulin. These two fractions also gave the highest dye-test titres and between them they contained most of the antibodies.

From these results it is seen that the highest dye-test titres are associated with fractions that contain γ-globulin. This suggests that the antibodies are associated with the presence of γ-globulin. The dye-test titres present in the other fractions could be assumed to be due to the presence of γ-globulin as a contaminant, which could be detected serologically as antibodies, but not electrophoretically under the conditions of these experiments. However, it should be pointed out that although fraction C contained about three-quarters of the γ-globulin, its antibody content was the same as that of fraction A, which did not contain as much γ-globulin. The explanation for this may be that the antibody molecules are associated with γ-globulin of a particular isoelectric value, which is more abundant in fraction A than in fraction C. On the other hand, if the antibodies are associated with the other possible protein, β-globulin, then it would be reasonable to expect a similar high titre in the other fraction which contains β-globulin, namely fraction B. Since this is not the case, it is evident that the cytoplasm-modifying antibodies are contained in the γ-globulin fraction of the serum proteins.

SUMMARY

Rabbit serum containing cytoplasm-modifying antibodies was fractionated by alcohol precipitation at low temperature and controlled ionic strength and pH. The highest cytoplasm-modifying antibody titres were observed in fractions that contained γ-globulin.

I am grateful to Dr R. Thomas of the Department of Biochemistry and Dr W. E. Ormerod of this Department for the loan of apparatus and for helpful suggestions. I am also indebted to the staff of the Department of Bacteriology for allowing me the use of their cold room and to Dr L. S. Yeh and Mr C. B. Hill of this Department for technical assistance. My grateful thanks are due to Prof. P. C. C. Garnham for his keen interest, helpful suggestions and criticisms during the course of this work.

REFERENCES

Cohn, E. J., Luetscher, J. A., Oncley, J. L., Armstrong, H. H. & Davis, B. D. (1940). Preparation and properties of serum and plasma proteins. III. Size and charge of proteins separating upon equilibration across membranes with ethanol-water mixtures of controlled pH, ionic strength and temperature. *J. Amer. Chem. Soc.* **62**, 3396–3400.

Conn, H. J. (1953). *Biological Stains. A Handbook on the Nature and Uses of the Dyes Employed in the Biological Laboratory*, 6th ed. Baltimore, The Williams & Wilkins Company.

Flynn, F. V. & de Mayo, P. (1951). Micro-electrophoresis of protein on filter-paper. *Lancet*, ii, 235–9.

Goldman, M. (1957*a*). Staining *Toxoplasma gondii* with fluorescein-labelled antibody. I. The reaction in smears of peritoneal exudate. *J. Exp. Med.* **105**, 549–56.

Goldman, M. (1957*b*). Staining *Toxoplasma gondii* with fluorescein-labelled antibody. II. A new serologic test for antibodies to *Toxoplasma* based upon inhibition of specific staining. *J. Exp. Med.* **105**, 557–73.

Kulasiri, C. (1960). The specificity of the Sabin-Feldman dye test with reference to protozoal infections. *J. Clin. Path.* (In the press.)

Nagaoka, R. (1957). A study of changes in serum protein of mice following an intraperitoneal inoculation of *Toxoplasma gondii*. *The Journal of the Osaka City Medical Centre*, **6**, 1037–50. (In Japanese. Read in abstract only.)

Wildführ, G., Naumann, G. & Wilde, J. (1958). Elektrophoretische Untersuchungen von Rattenseren nach Infektion mit *Toxoplasma gondii*. *Z. ImmunForsch.* **115**, 122–34.

Parasitology (1960), **50**, 425–429
With 11 *text-figures*
Printed in Great Britain

Two new species of trichostrongyle nematodes from the Australian pouched mouse, *Phascogale flavipes* (Waterhouse)

By PATRICIA M. MAWSON, M.Sc.
Department of Zoology, University of Adelaide, South Australia

(*Received* 30 *November* 1959)

The nematodes described below were collected by M. L. Emanuel from two specimens of the pouched mouse, at Innisfail, North Queensland. They were very kindly sent to the author by Dr M. J. Mackerras of the Queensland Institute of Medical Research. Both species of parasite were present in both host animals. Trichostrongyles have not previously been described from phascogales but a metastrongyle, two spirurids, and a rictulariid, are listed by Dr Mackerras in her check-list of parasites of Australian mammals (Mackerras, 1958). To these may now be added the present records, the details of which are as follows: *Phascogale flavipes* (Waterhouse, 1838). Innisfail, Queensland. *Nicollina phascogale* sp.nov., *Tetrabothriostrongylus mackerrasae* gen.nov., sp.nov.

Types. The holotype male and allotype female of both species described have been deposited in the South Australian Museum. Paratype material is in the Helminthological Collection of the Zoology Department of the University of Adelaide and in the collection of the Queensland Institute of Medical Research, Brisbane.

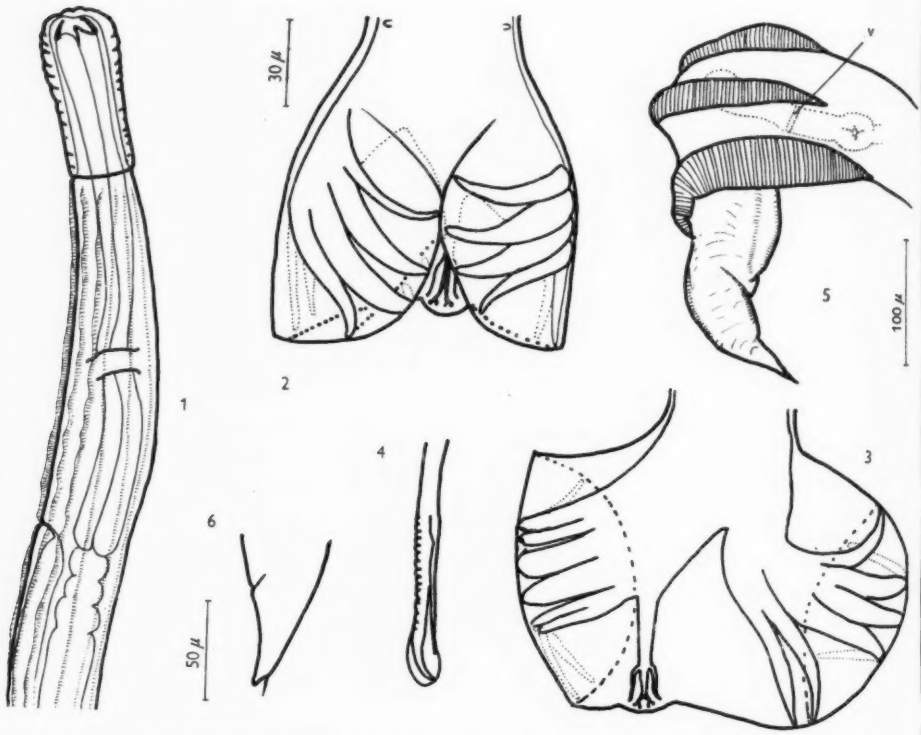
Nicollina phascogale sp.nov. (Figs. 1–6)

This species is the smaller of the two described here; the body in most specimens is coiled into a tight spiral. The cephalic cuticle is inflated and coarsely annulated. The cuticle over the rest of the body is more finely annulated and is raised into lateral alae and four pairs of longitudinal ridges of which the ventral four are the wider; these ridges extend nearly to the bursa in the male, and nearly to the vulva in the female. The buccal capsule contains a dorsal tooth, the length of which is about half that of the buccal cavity. The oesophagus is narrow throughout its length; the excretory pore lies near the base of the oesophagus; the nerve ring has been seen in only one specimen, lying at about two-thirds the length of the oesophagus from the anterior end (Fig. 1).

In the female, the body is wider in the vulvar region, and four alae, in submedian positions (but not continuous with the longitudinal ridges over the rest of the body) are greatly thickened and widened. The vulva is a transverse slit. The ovijectors are short, of equal length; no eggs are present. The tail is short, conical, with rounded tip, and bears a short dorsal subterminal spine (Figs. 5, 6).

In the male the spicules are relatively short and complex; the distal third of each is split so that each ends in a long curved part with a blunt tip and a shorter

pointed piece, these two furcae supporting a rounded terminal ala. The gubernaculum is poorly chitinized. The bursa is so rolled that it proved impossible to get a good drawing with the lateral lobes outspread; the lateral lobes are long, the dorsal small; one side is longer than the other and the dorsal ray arises from the base of the ventral and lateral rays on this side. On the inside the bursa is lined with tiny cuticular striae so that the rays are difficult to see. Their arrangement is shown in Figs. 2 and 3.



Figs. 1–6. *Nicollina phascogale*. 1, Anterior end of male; 2, ventral, and 3, dorsal, views of bursa; 4, tip of spicule; 5, posterior end of female, twisted but mostly in dorsal view; 6, tail of female. Figs. 2 and 3 to same scale; Figs. 1, 4, and 6 to same scale. *v*, vulva.

The measurements of the male and female respectively are as follows: length of body, 3·1–5·0 mm., 4·2–5·6 mm.; of oesophagus 230–300μ, 320–340μ; of cephalic inflation 65–80μ (1/3·3–1/4 that of oesophagus), 60–90μ (1/3·5–1/4); length of spicules 200–250μ (1/16–1/20 of body length), and of gubernaculum about 50μ (1/5 spicule length); the distance of the vulva from the posterior end (1/16–1/20 of body length) is 250–300μ; the tail length is 40–60μ (1/6·5–1/7·5 of distance from vulva to posterior end).

The presence of two lateral alae and eight longitudinal ridges distinguishes the species from all others of the genus except *N. tachyglossae* Baylis (1930) and *N. iota*

Mawson (in press). It differs from these in the shape of the bursa and of the female tail, the branching of the dorsal ray, and in the presence of four swollen alae in the vulvar region.

Tetrabothriostrongylus mackerrasae gen.nov., sp.nov. (Figs. 7–11)

This interesting new species was taken from two specimens of *Phascogale flavipes* from Innisfail.

The worms are of nearly even width throughout, tapering in the oesophageal region. The males are 3·7–4·0 mm. long, the females 5·4–7·6 mm. The head is bent dorsad; the cephalic cuticle is inflated and forms four epaulette-like structures around the anterior end, arranged symmetrically in submedian positions. These projections are purely cuticular and each is supported by a very thick triangular rim (Figs. 7, 8). No circumoral papillae, or amphids, were seen. The cuticle over the rest of the body is striated transversely and longitudinally. Two narrow lateral alae are present, but other longitudinal ridges are not definite or continuous.

There is no buccal capsule. The oesophagus is narrow for the first fifth of its length, then widens suddenly; at about the level of the widening lie the nerve ring excretory pore, and small cervical papillae. The length of the oesophagus is 500–690μ in the male and 500–800μ in the female; the distance of the excretory pore from the anterior end in the female is 160–250μ (Fig. 7).

The spicules, 150–190μ long, are relatively short, and from the mid-length divided into three more or less equal and pointed branches. A gubernaculum 70–90μ long is present. The bursa is wide and symmetrical; the arrangement of the rays is shown in Figs. 10 and 11.

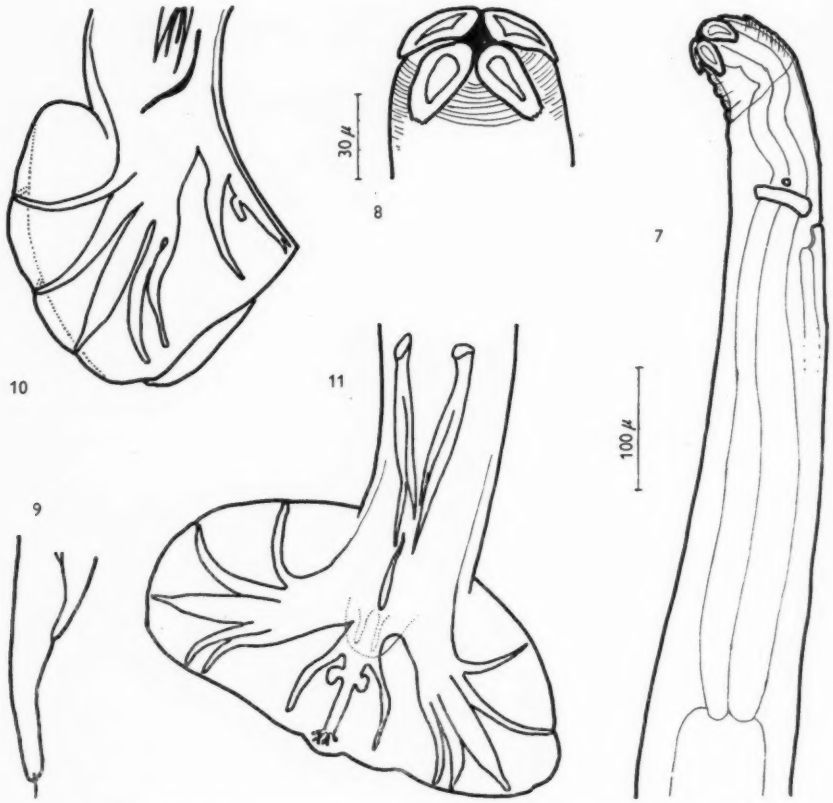
The female tail, 100–140μ long, is finger-like, rounded at the tip and bears a short spike (Fig. 9). The vulva, a transverse slit without prominent lips, lies 1·3–1·7 mm. from the posterior end. The eggs are 60–70μ by 40–45μ.

The species is distinguished from all other trichostrongyles by the presence of four cuticular expansions around the head as well as of a gubernaculum. A new genus is proposed for it, *Tetrabothriostrongylus*, of which the diagnosis is as follows:

TRICHOSTRONGYLIDAE: cuticle with transverse striae and narrow lateral alae; cephalic cuticle inflated, with four triangular cuticular projections in submedian positions around mouth; no tooth or buccal capsule; female tail digitiform with terminal spike, vulva about a quarter to a fifth of body length from posterior end; male with relatively short three branched non-alate spicules, gubernaculum present; bursa large, ventral and lateral rays long, all more or less divergent from one another, externo-dorsal ray arising from dorsal, dorsal giving off one pair of branches before bifurcation. Type, *T. mackerrasae* sp.nov.

The appearance of the head of this species suggests that of *Epomidiostomum* spp. However the development of four cuticular processes in the cephalic cuticle is also comparable with that of the two ventral hooks in *Biacantha desmoda* Wolfgang, of the three ventral hooks in *Asymmetracantha tasmaniensis* Mawson (in press), of the backwardly projecting 'frill' in *Tricholeiperia* spp. and *Anoplostrongylus*

paradoxus (Travassos), and of the 'ears' in *Torrestrongylus torrei* Viguerras. It can be regarded as an example of a type of modification likely to be developed in any group with inflated cuticle at the anterior end, and is a convergent rather than an homologous feature.



Figs. 7–11. *Tetrabothriostrongylus mackerrasae*. 7, Anterior end of female; 8, head in ventral view; 9, tail of female; 10, lateral, and 11, dorsal, views of bursa. Figs. 7 and 9–11, to same scale.

The new species differs from *Epomidiostomum* spp. in the presence of a gubernaculum in the male, and of a spine on the female tail. Apart from the cephalic ornamentation, it falls (in the classification of Skrjabin, Shikhobalova, Popova, Boev & Delyamure, 1952) in the subfamily Molineinae Skrjabin & Schulz, and it agrees generally with genera in this group in the characters of spicules, bursa and female tail. It also has these characters in common with some genera allotted by the same authors to the subfamily Citellinematinae.

REFERENCES

BAYLIS, H. A. (1930). Four new trichostrongyle nematodes from Queensland. *Ann. Mag. nat. Hist.* ser. 10, **6**, 1–18.

MACKERRAS, M. J. (1958). Catalogue of Australian mammals and their recorded internal parasites. I–IV. Pt. 1, Monotremes and Marsupials. *Proc. Linn. Soc. N.S.W.* **83**, 101–25.

MAWSON, P. M. (1960). Nematodes belonging to the Trichostrongylidae, Subuluridae, Rhabdiasidae, and Trichuridae from bandicoots. *Austr. Jour. Zool.*, (in press).

SKRJABIN, K. I., SHIKHOBALOVA, N. P., POPOVA, T. I., BOEV, S. N. & DELYAMURE, S. L. (1952). *Descriptive Catalogue of Parasitic Nematodes*, Vol. 3, Strongylata, 890 pp. Moscow. (In Russian.)

WOLFGANG, B. W. (1954). Studies on the endoparasitic fauna of Trinidad mammals. X. Parasites of Chiroptera. *Canad. J. Zool.* **32**, 20–4.

Parasitology (1960), **50**, 431–448
Printed in Great Britain

Further observations on the *in vitro* development of the gametocytes of *Plasmodium gallinaceum*

By ANN BISHOP and ELSPETH W. McCONNACHIE

Members of the scientific staff of the Medical Research Council. Molteno Institute, University of Cambridge

CONTENTS

I. Introduction 431
II. General methods 432
III. The effect of horse serum and rabbit plasma upon the development of the gametocytes of *Plasmodium gallinaceum* 433
IV. The effect of immune plasma upon the *in vitro* development of the gametocytes of *Plasmodium gallinaceum* 435
V. The effect of various inorganic ions upon the *in vitro* development of the gametocytes of *Plasmodium gallinaceum* 436
VI. Discussion 442
VII. Summary 444
References 444
Appendix 445

I. INTRODUCTION

The gametocytes of plasmodia do not emerge from the erythrocytes whilst in the bloodstream of the vertebrate host, but if the infected blood is ingested by susceptible mosquitoes they leave the corpuscles and gametes are formed in the stomach of the insect. The formation of the motile, threadlike male gametes from the male gametocyte is a striking phenomenon and is known as exflagellation. The gametocytes also emerge from the corpuscles and exflagellation occurs in blood withdrawn artificially from the vertebrate host, provided that the blood is exposed to air.

Marchoux & Chorine (1932) who worked on *Haemoproteus*, found that these changes took place over a wide range of temperature (13–43 °C.). They observed, however, that exposure of the gametocytes to CO_2 prevented their emergence from the corpuscles; this inhibition could be reversed by subsequently exposing the blood to air, provided that the exposure to CO_2 had not been too prolonged. Similar results were obtained by Chorine (1933) with *Plasmodium praecox* (*P. falciparum*). This work was repeated, using *P. gallinaceum*, by Bishop & McConnachie (1956), who obtained satisfactory recovery from the inhibitory effect of the gas only after exposure to 5% CO_2 in 95% O_2 or N_2, recovery from 100% CO_2 being more variable.

Marchoux & Chorine believed that the actual factor initiating the emergence of the gametocytes from the corpuscles was a change in the pH of the blood due to the loss of CO_2, though Chorine (1933) pointed out that this probably only triggered off a series of more complex reactions in the blood which brought about the changes in the gametocytes. Bishop & McConnachie recorded a rise in the pH of freshly

drawn, normal and infected chick blood exposed to air, but, in experiments with infected blood, in which this rise in pH was inhibited, they were unable to confirm that pH is the critical factor controlling the development of the gametocytes. Moreover, gametocytes did not emerge from the corpuscles if these were washed and suspended in isotonic saline buffered to pH 7·78, a pH at which the emergence of gametocytes readily takes place in blood, but they subsequently emerged in large numbers and the males exflagellated when the saline was replaced by chicken plasma; this suggested that some factor or factors present in chick plasma were necessary for the development of the gametocytes.

The following experiments were carried out in order to obtain further knowledge of the conditions under which gametocytes of malaria parasites emerge from the corpuscles and exflagellation of the male gametes takes place.

II. GENERAL METHODS

In the following experiments, as in those described previously (Bishop & McConnachie, 1956), a strain of *P. gallinaceum* was used which was maintained by mosquito passage through chicks 5 days to 5–6 weeks old. Although the intensity and duration of acute infection is less in infections thus induced than in infections produced by the inoculation of infected blood, the proportion of gametocytes to asexual parasites is usually much greater. Only blood containing large numbers of mature gametocytes was used in the experiments.

Shortly before they were bled, the birds were injected intravenously with heparin (0·1 % in saline), the standard dose being 0·05 mg./20 g. body weight. The infected blood was withdrawn from the jugular vein by syringe, care being taken to exclude bubbles of air. The blood was immediately ejected from the syringe into the fluids to be tested, contained in centrifuge tubes. Unless otherwise stated, 0·5 ml. of heavily parasitized blood was added to 4·5 ml. of the test fluid. The parasitized corpuscles were immediately spun down, the supernatant fluid removed, and the corpuscles resuspended in 4·5 ml. of the test fluid. The corpuscles were kept in suspension by frequent stirring.

Comparisons of the numbers of gametocytes emerging from the erythrocytes and of males exflagellating in the different media were made by microscopic inspection of the living organisms in drops under coverslips, samples of the suspensions of parasitized corpuscles being removed at intervals of 4–5 min. for examination. The first examination was made 10–15 min. after the withdrawal of the blood, and exflagellation normally took place within 20 min. of bleeding. Examinations were continued for at least an hour. No satisfactory method was discovered of counting the exact numbers of gametocytes emerging and of males exflagellating, but preparations were examined by both workers independently, and the results scored as (+), +, ++, +++. When only one or two exflagellating individuals were seen the result was recorded as $(+)^1$, or $(+)^2$.

All experiments were carried out at room temperature, which varied from 18 to 25 °C. Glassware, including slides and coverslips, was of hard glass, either Pyrex or Chance. The salt solutions were made up from 'Analar' Reagents

(British Drug Houses Ltd.) in water freshly distilled in a glass still. Sigma 7–9 Biochemical buffer Tris (hydroxymethylaminomethane), with an approximate range of pH 7–9 (Sigma Chemical Company, St Louis, U.S.A.) was incorporated in all the salt solutions, and the pH adjusted, with N/10 HCl or 10% H_2SO_4, to approximately 8·0, a pH at which the gametocytes readily develop in blood (Bishop & McConnachie, 1956). The concentrations of salts and buffer were such that all the solutions used were isotonic with bird blood (Heilbrunn, 1952).

III. THE EFFECT OF HORSE SERUM AND RABBIT PLASMA UPON THE DEVELOPMENT OF THE GAMETOCYTES OF *PLASMODIUM GALLINACEUM*

The gametocytes of *Plasmodium gallinaceum* developed normally when parasitized corpuscles were washed and suspended in normal chick plasma, chick plasma inactivated at 56 °C. or Seitz-filtered chick serum. In contrast, no change was observed to occur in the gametocytes when the corpuscles were washed and suspended in saline buffered to a favourable pH, but they emerged from the corpuscles and the males exflagellated when the saline was subsequently replaced by chick plasma (Bishop & McConnachie, 1956). In order to discover whether the stimulating action of chick plasma and serum on the gametocytes was specific, the effect of plasma and serum from other vertebrates was studied.

Normal chick plasma was prepared by centrifuging blood from an uninfected chicken previously injected with heparin. In a series of experiments, the development of the gametocytes was compared in normal chick plasma or serum, normal, Seitz-filtered horse serum, Seitz-filtered horse serum inactivated at 56 °C. and saline (see Appendix, solution I). Few gametocytes emerged from the corpuscles and exflagellation was rare in saline, but in normal or inactivated horse serum the number of gametocytes emerging from the corpuscles and the frequency of exflagellating male gametocytes appeared to be greater even than in normal chick plasma (Table 1, Expts. 1–4). It was also noticed that exflagellation did not continue for as long in the horse serum as in the chick plasma or serum, and the impression that greater numbers of exflagellating males occurred in the horse serum was probably due to their earlier and more simultaneous development. Both normal and inactivated horse serum had a very strong agglutinating action upon both corpuscles and free parasites, which stuck together to form large masses.

In these experiments with horse serum the parasitized blood was diluted 1 in 10. In higher dilutions the density of suspended corpuscles is so low that it is not easy to make a reliable comparison of the numbers of the gametocytes without further concentration by centrifugation, which increases the time before the first microscopic observation can be made. It was possible, however, that in a dilution of 1 in 10 the proportion of chick plasma introduced into the horse serum with the corpuscles might have been large enough, before centrifugation and removal of the supernatant fluid, to initiate the changes in the gametocytes even though it was unable to do so in saline. In a further experiment, therefore, 0·1 ml. of heavily parasitized blood was added to 5 ml. of horse serum, giving a dilution of 1 in 51. After centrifugation and removal of the supernatant, the corpuscles were sus-

pended in 5 ml. of normal horse serum and concentrated by centrifugation in order to facilitate examination. Large numbers of exflagellating male gametocytes were observed amongst the corpuscles.

Table 1. *The effect of horse serum, saline and a balanced salt solution on the exflagellation of the male gametocytes of* Plasmodium gallinaceum

	Exflagellation					
No. of expt.	Normal chick plasma	Normal chick serum	Normal horse serum	Inactivated horse serum	Solution I*	Solution II*
1	++	–	+++ pH 8·23	–	(+)[2] pH 8·20	–
2	+(+)	–	–	++(+)	(+)[1] pH 8·20	–
3	++	–	+++	–	–	–
4	+(+) pH 7·78	–	+++ pH 7·68	–	–	+++ pH 8·15
5	–	+++ pH 8·05	–	–	–	+(+) pH 8·40
6	–	+++ pH 7·99	–	–	–	++ pH 8·00

* See Appendix.

See p. 432 for the method of recording, in Tables 1–6, the relative numbers of gametocytes emerging and exflagellating.

In a similar experiment the effect of horse serum and rabbit plasma upon the development of the gametocytes was compared. Blood was removed from a rabbit's ear, and 2 mg. of heparin in 2 ml. of normal saline was added to 25 ml. of the blood. The plasma was removed from the corpuscles by centrifugation. 0·1 ml. of parasitized blood was added to each of two centrifuge tubes, one containing 5 ml. of horse serum and the other 5 ml. of rabbit plasma. After centrifugation the supernatant was removed and the packed corpuscles were resuspended in 5 ml. of fresh fluid. The corpuscles were concentrated by centrifugation, and large numbers of exflagellating male gametocytes were seen both in the rabbit plasma and the horse serum.

Since we had the impression that in horse serum the gametocytes emerged from the erythrocytes sooner than in normal chick plasma, and that the development of the exflagellating males was more simultaneous but came to an end sooner than in chick plasma, it seemed possible that some factor in horse serum might prevent the gametes from completing their development. To test this, parasitized corpuscles suspended in horse serum were fed to *Aëdes aegypti* through a membrane by the method devised by Bishop & Gilchrist (1946). 1·5 ml. of parasitized blood was diluted 1 in 10 in normal horse serum, after which the corpuscles were concentrated by centrifugation and the supernatant fluid removed. The corpuscles were resuspended in 1·5 ml. of horse serum and the suspension warmed to 40° C., poured into a membrane apparatus with a water-jacket at the same temperature, and fed to the mosquitoes in an incubator at 28 °C. Within 30 min. most of the mosquitoes had become fully engorged. Four mosquitoes were killed

and examined on the 4th day following feeding, and six on the 6th day. All had oocysts upon the stomach wall, the number varying from 7 to 37, with a mean of 20·5. The appearance of all the oocysts was normal. The strongly agglutinating action of the horse serum changed the physical character of the suspension of corpuscles drastically as compared with normal blood, so that although the mosquitoes were able to ingest the normal volume of serum, the clumping of the corpuscles prevented them from ingesting corpuscles in normal numbers. No attempt was therefore made to compare the intensity of infections produced in mosquitoes fed upon the suspension of parasitized corpuscles in horse serum with those in mosquitoes fed on parasitized chick blood. The experiment, however, proved conclusively that gametocytes suspended in normal horse serum were able to complete their sexual development and were infective to mosquitoes.

IV. THE EFFECT OF IMMUNE PLASMA UPON THE *IN VITRO* DEVELOPMENT OF THE GAMETOCYTES OF *PLASMODIUM GALLINACEUM*

Lumsden & Bertram (1940) noticed that the maximum infectivity of gametocytes of *Plasmodium gallinaceum* to *Aëdes aegypti* did not always coincide with the maximum gametocyte production. This observation was confirmed by Cantrell & Jordan (1946) who showed that the infectivity of the gametocytes reached a peak on the day before the peak of parasitaemia and thereafter declined rapidly. It was further observed that serum from heavily infected chickens at the peak of parasitaemia caused a decrease in the infectivity of gametocytes from an earlier stage of infection, when these gametocytes were exposed to the serum and subsequently fed to *A. aegypti* through a membrane (Eyles, 1952*b*). More recently, Huff & Marchbank (1955) carried out a study of the infectivity of gametocytes of *Plasmodium gallinaceum*, *P. fallax* and *P. cathemerium* to mosquitoes at different periods during the course of infection in the vertebrate host. They found that maximum oocyst production occurred in mosquitoes which had been fed 1 to 4 days before the peak of parasitaemia and that there was a precipitous fall in oocyst numbers in mosquitoes fed upon hosts at a slightly later stage of infection, even though the total number of parasites, including gametocytes, was still increasing. As a result of further experiments, Huff, Marchbank & Shiroishi (1958) suggested that this decrease in infectivity of gametocytes might be due to active immunity developing in the vertebrate host.

The following experiments were undertaken in order to discover whether plasma from hyperimmune birds had any inhibitory effect upon the *in vitro* development of the gametocytes. The donor birds (Table 2, nos. 71, 114, 617) had been hyperimmunized, after the decline of the primary infection, by the intravenous injection of 1·5–8 ml. of heavily infected blood. Bird no. 71 was superinfected five times during the course of the experiments, bird no. 114 four times, and bird no. 617 three times. In each experiment the donor bird received an injection of heavily parasitized blood several days before the removal of blood for the production of plasma. The clearance of parasites from the blood following superinfection was, in every case, rapid, indicating that a high degree of immunity had developed. The immune plasma was obtained shortly before use by bleeding a bird which

had been injected with the standard dose of heparin, the plasma being separated from the corpuscles by centrifugation. For the control experiments plasma was similarly obtained from heparinized, uninfected birds.

Agglutination of the corpuscles was observed in the presence of the hyperimmune plasma, as had previously been observed in *P. knowlesi* (Eaton, 1938) and in *P. gallinaceum* (Zuckerman, 1945). Gametocytes emerged from the erythrocytes suspended in hyperimmune plasma and were normal in appearance, and exflagellation of the male gametes occurred. No significant difference was observed in the time of onset or the frequency of exflagellation in gametocytes in hyperimmune plasma as compared with those in normal plasma (Table 2).

Table 2. *The effect of immune chick plasma on the* in vitro *development of the gametocytes of* Plasmodium gallinaceum

No. of expt.	Plasma	Emergence of gametocytes	Exflagellation
1	Immune from bird 71	(+)	+(+)
	Normal	−	+++
2	Immune from bird 71	+	+(+)
	Normal	++	+++
3	Immune from bird 71	+	+
	Normal	(+)	+
4	Immune from bird 71	+	++(+)
	Normal	+	++
5	Immune from bird 114	+++	+++
	Normal	+++	+++
6	Immune from bird 617	+	++
	Normal	(+)	(+)

No attempt was made to compare the infectivity to mosquitoes of gametocytes treated with hyperimmune plasma with those treated with normal plasma since, as in the experiments with horse serum, the agglutination of the corpuscles introduced a mechanical factor which renders the results not strictly comparable.

V. THE EFFECT OF VARIOUS INORGANIC IONS UPON THE *IN VITRO* DEVELOPMENT OF THE GAMETOCYTES OF *PLASMODIUM GALLINACEUM*

The results of experiments with horse serum and rabbit plasma showed that the emergence of the gametocytes of *P. gallinaceum* from the erythrocytes and the subsequent development of the male gametes could take place normally in media other than chick plasma or serum. Since, however, it had been found previously (Bishop & McConnachie, 1956) and confirmed later (Table 1, expts. 1, 2) that few or no gametocytes emerged from the corpuscles and exflagellation

was rare when parasitized erythrocytes were washed and suspended in isotonic sodium chloride buffered to a favourable pH (solution I), the effect of other salts upon these processes was studied.

The first solution (solution II) to be studied contained NaCl, KCl, $CaCl_2$, $NaHPO_4$, $MgSO_4$, $NaHCO_3$ and Tris buffer, the pH being adjusted to approximately 8 with N/10 HCl or 10% H_2SO_4 (see Appendix). Exflagellation occurred in solution II and, as in chick plasma and horse or chick serum, the appearance of the free gametocytes and the movements of the exflagellating males were normal (Table 1, Expts. 4–6). The development of the gametocytes proceeded equally well in solutions whose pH had been adjusted with hydrochloric or sulphuric acid. As had been observed previously in whole blood (Bishop & McConnachie, 1956), exflagellating gametocytes were first seen 15–20 min. after the withdrawal of the blood from the host.

Since the gametocytes appeared to develop as well in this mixture of inorganic salts as in horse serum, or plasma or serum from the normal host, experiments were performed in order to discover which ions were essential for their development.

Solution II contained the following ions—Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HPO_4^-, HCO_3^- and SO_4^{2-}. A series of solutions was prepared, in which one or two of the ions present in solution II were omitted, and the development of the gametocytes in these solutions was compared with controls in the complete solution II. The emergence of the gametocytes from the erythrocytes and the subsequent exflagellation of the males was not affected by the absence of Mg^{2+}, and SO_4^{2-}, HPO_4^-, K^+ or Ca^{2+} (Table 3, Expts. 1–8). The gametocytes did not, however, develop normally in the absence of Na ions (solution VII); although they emerged from the erythrocytes, the numbers of males exflagellating were fewer than in the controls and their movements feeble (Table 3, Expts. 9, 10). The development of the gametocytes was almost completely inhibited when HCO_3 ions were absent (solution VIII); gametocytes rarely emerged from the erythrocytes and only one exflagellating male was seen in three experiments (Table 3, Expts. 11–13). In two experiments, after the parasitized corpuscles had been suspended for 30 min. in the solution without HCO_3 ions, the corpuscles were concentrated by centrifugation and the supernatant fluid removed. Approximately half the corpuscles were resuspended in the same fluid and the other half were suspended in solution II. Whereas no gametocytes emerged from the corpuscles in the solution devoid of HCO_3 ions, in solution II many emerged though the males failed to exflagellate. The inhibitory effect of the absence of HCO_3 ions was therefore only partially reversible after a period of 30 min.

Since few or no gametocytes emerged from the erythrocytes and exflagellating males were rare in buffered saline, and since the presence of Na and HCO_3 ions was essential for normal development in the balanced salt solution II, the effect of adding $NaHCO_3$ to NaCl (solution IX) was studied. Under these conditions, free gametocytes were numerous and the numbers of males exflagellating were as great as in the controls (Table 4, Expts. 1–4).

These experiments indicated that gametocytes could emerge from the corpuscles and the males exflagellate normally in the presence of only Na, Cl and HCO_3 ions.

In order to test the effect of omitting Cl ions, a solution of $NaHCO_3$ was prepared in which the pH was adjusted with 10% H_2SO_4 (solution X). In this solution the gametocytes emerged from the erythrocytes in numbers comparable to the controls,

Table 3. *The effect upon the development of the gametocytes of* Plasmodium gallinaceum *of the omission of certain ions from solution II*

Solution*	Ions present +ve	Ions present −ve	Ions missing +ve	Ions missing −ve	Expt.	Emergence of gametocytes	Exflagellation
II†					1	++	+++
					2	++	+++
III	Na, K, Ca	Cl, HPO_4, HCO_3	Mg	SO_4	1	+	+++
					2	++	+++
II					3	++	+++
					4	++	+++
IV	Na, K, Ca, Mg	Cl, SO_4, HCO_3		HPO_4	3	++	+++
					4	++	+++
II					5	++	+++
					6	++	+++
V	Na, Ca, Mg	Cl, HPO_4, SO_4, HCO_3	K		5	++	+++
					6	++	+++
II					7	+	+(+)
					8	++	++
VI	Na, K, Mg	Cl, HPO_4, SO_4, HCO_3	Ca		7	+	+(+)
					8	++	++
II					9	++	+++
					10	++	++(+)
VII	K, Ca, Mg	Cl, HPO_4, SO_4, HCO_3	Na		9	++	++f
					10	+(+)	(+)f
II					11	++	+++
					12	+	+(+)
					13	++	+++
VIII	Na, K, Ca, Mg	Cl, HPO_4, SO_4		HCO_3	11	(+)[2]	0
					12	(+)[2]	0
					13	0	(+)[1]

* See Appendix.

† Solution II contained the following ions: Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HPO_4^-, HCO_3^- and SO_4^{2-}.

f = movements of male gametes feeble.

Table 4. *The effect upon the development of the gametocytes of* Plasmodium gallinaceum *of certain combinations of inorganic ions* (Na^+, Cl^- *and* HCO_3^-)

Solution*	Ions present +ve	Ions present −ve	Ions missing +ve	Ions missing −ve	Expt.	Emergence of gameto-cytes	Exflagellation
II†					1	++	+++
					2	++	++
					3	++	+++
					4	+++	+++
I	Na	Cl	K, Ca, Mg	HPO_4, SO_4, HCO_3	1	(+)	+(+)
					2	(+)	(+)
					3	(+)	(+)[1]
					4	(+)[3]	(+)[2]
IX	Na	Cl, HCO_3	K, Ca, Mg	HPO_4, SO_4	1	+	+++
					2	++	++
					3	+(+)	+++
					4	−	+++
II					5	+	+(+)
					6	+++	+++
					7	+	++
					8	+++	+++
					9	+(+)	+
X	Na	HCO_3, [SO_4]	K, Ca, Mg	HPO_4, Cl	5	+(+)	(+)f
					6	+++	(+)[1]f
					7	++	(+)f
					8	+++	(+)f
					9	+	(+)f
II					10	+++	+++
					11	+(+)	+
					12	++	+++
					13	+++	+++
					14	+	++(+)
					15	(+)	+
					16	++	+++
					17	++	+++
X	Na	HCO_3, [Cl]	K, Ca, Mg	HPO_4, SO_4	10	+++	++f
					11	+	+f
					12	++	+++
					13	++(+)	+(+)f
					14	(+)	(+)f
					15	+	(+)f
					16	+++	(+)f
					17	++	+(+)f

* See Appendix.

† Solution II contained the following ions: Na^+, K^+, Ca_2^+, Mg^{2+}, Cl^-, HPO_4^-, HCO_3^- and SO_4^{2-}.

f = movements of male gametes feeble.

[ ] − ions present in very low concentration, in acid used to adjust pH of solution.

but few male gametocytes exflagellated, their movements were feeble and their appearance abnormal (Table 4, Expts. 5–9). It seems, therefore, that in the presence of Na and HCO_3 ions, Cl ions are also necessary for the normal development of the gametocytes.

Further evidence in support of this was obtained from experiments in which the only Cl ions present were those in the acid (N/10 HCl) used to adjust the pH of the $NaHCO_3$ solution. In seven out of eight experiments significantly fewer exflagellating males were seen in the buffered $NaHCO_3$ than in the controls, and their movements were feeble (Table 4, Expts. 10, 11, 13–17). In one experiment, however, (Expt. 12) the number of males exflagellating was as great as in solution II, and their movements were normal. It is possible that in this experiment sufficient HCl was added to the $NaHCO_3$ solution to provide the concentration of Cl required by the gametocytes in the presence of Na and HCO_3 ions.

Experiments were carried out to determine whether the gametocytes could develop in minimal combinations of ions other than Na, Cl and HCO_3. A solution containing only K and Cl ions (solution XV) completely inhibited the emergence of the gametocytes and the exflagellation of the males (Table 5, Expts. 1–4). In the presence of K, HCO_3 and a small proportion of Cl ions (solution XVII), the gametocytes emerged from the erythrocytes in numbers comparable with those in the controls, but no exflagellating males were seen, and similar results were obtained when KCl was added to the $KHCO_3$ solution (solution XVIII) (Table 5, Expts. 3, 4). Thus K ions cannot replace the Na ions necessary, in the presence of Cl and HCO_3 ions, for normal exflagellation.

The addition of $NaHCO_3$ to a solution of KCl (solution XVI) could provide conditions in which the gametocytes emerged and exflagellated as well as they did in the control solution II (Table 5, Expts. 1, 2). Thus normal exflagellation occurred in a solution containing Na, K, HCO_3 and Cl ions, in which the proportion of KCl to $NaHCO_3$ was 4–21. These four ions were also provided by a different combination of salts—NaCl and $KHCO_3$, instead of KCl and $NaHCO_3$. In solution XXI, containing 4 parts of NaCl to 21 parts of $KHCO_3$, the gametocytes emerged from the corpuscles in numbers comparable to those in the controls, but no exflagellation took place (Table 5, Expts. 5, 6). If, however, the solution contained 21 parts of NaCl to 4 parts of $KHCO_3$ (solution XXII), the development of the gametocytes proceeded as in the controls (Table 5, Expt. 6). Thus an excess of K over Na ions inhibited the exflagellation of the male gametes, whereas variations in the proportions of Cl and HCO_3 ions had no effect.

In three experiments in which the parasitized corpuscles were washed and suspended in a solution containing Na, HPO_4 and a small proportion of SO_4 ions (solution XI), gametocytes emerged from the corpuscles but the males did not exflagellate (Table 6, Expts. 1–3). If the solution also contained HCO_3 ions (solution XII), although a few males exflagellated, their numbers were very small compared with those in solution II and their movements were feeble (Table 6, Expts. 1–3).

In a solution containing only Mg and SO_4 ions (solution XIII), gametocytes emerged from the erythrocytes but exflagellation, if it occurred at all, was much

less frequent than in the controls and the movements were feeble (Table 6, Expts. 4–6). The numbers of males exflagellating did not increase when, in addition to the Mg and SO_4 ions, the solution also contained Na and HCO_3 ions (solution XIV).

In a solution containing only Ca and Cl ions (solution XIX), the numbers of gametocytes emerging from the erythrocytes were comparable to those in solution II, but exflagellating males were either absent or few in number, and feeble in movement. When $NaHCO_3$ was added to the solution of $CaCl_2$ (solution XX) to produce a solution containing Ca, Na, Cl and HCO_3 ions, though the number of

Table 5. *The effect upon the development of the gametocytes of* Plasmodium gallinaceum *of certain combinations of inorganic ions* (K^+, Cl^-, HCO_3^- *and* Na^+)

	Ions present		Ions missing			Emergence of gameto-	
Solution*	+ve	−ve	+ve	−ve	Expt.	cytes	Exflagellation
II†					1	+	++(+)
					2	(+)	+
XV	K	Cl	Na, Ca, Mg	HCO_3, HPO_4, SO_4	1	0	0
					2	0	0
XVI	Na, K	HCO_3, Cl	Ca, Mg	HPO_4, SO_4	1	(+)	++
					2	+	+(+)
II					3	++	+++
					4	++	++(+)
XV	K	Cl	Na, Ca, Mg	HCO_3, HPO_4, SO_4	3	(+)[1]	0
					4	(+)[2]	0
XVII	K	HCO_3, [Cl]	Na, Ca, Mg	HPO_4, SO_4	3	++	0
					4	++	0
XVIII	K	HCO_3, Cl	Na, Ca, Mg	HPO_4, SO_4	3	++	0
					4	++	0
II					5	+++	+++
					6	++	++
I	Na	Cl	K, Ca, Mg	HCO_3, HPO_4, SO_4	5	(+)	(+)[1]
					6	(+)[2]	0
XVII	K	HCO_3, [Cl]	Na, Ca, Mg	HPO_4, SO_4	5	+++	0
					6	++	0
XXI‡	Na, K	Cl, HCO_3	Ca, Mg	HPO_4, SO_4	5	+++	0
					6	++	0
XXII‡	Na, K	Cl, HCO_3	Ca, Mg	HPO_4, SO_4	6	+	++

* See Appendix.

† Solution II contained the following ions—Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HPO_4^-, HCO_3^- and SO_4^{2-}.

‡ Solutions XXI and XXII contained different proportions of the same salts.

[ ] −ions present in very low concentration, in acid used to adjust pH of solution.

exflagellating males was somewhat larger than in the solutions in which only Ca and Cl ions were present, in one of the two experiments their movements were feeble (Table 6, Expts. 7, 8). In these experiments no estimations have been made of the free HCO_3 present, and it is possible that fewer HCO_3 ions were present in solution XX owing to the relative insolubility of Ca carbonates.

Table 6. *The effect upon the development of the gametocytes of* Plasmodium gallinaceum *of certain combinations of inorganic ions* (Na^+, HPO_4^-, HCO_3^-, Mg^{2+}, SO_4^{2-}, Ca^{2+} *and* Cl^-)

Solution*	Ions present +ve	Ions present −ve	Ions missing +ve	Ions missing −ve	Expt.	Emergence of gametocytes	Exflagellation
II†					1	+	+(+)
					2	+	+++
					3	++	+++
XI	Na	HPO_4, $[SO_4]$	K, Ca, Mg	Cl, HCO_3	1	+	0
					2	+	0
					3	+	0
XII	Na	HPO_4, HCO_3, $[SO_4]$	K, Ca, Mg	Cl	1	+	(+)f
					2	+	(+)f
					3	+(+)	+f
II					4	+	+(+)
					5	+++	+++
					6	+	++
XIII	Mg	SO_4	Na, K, Ca	HPO_4, Cl, HCO_3	4	+	0
					5	++	+(+)f
					6	(+)	(+)[2]
XIV	Mg, Na	SO_4, HCO_3	K, Ca	HPO_4, Cl	4	++	(+)f
					5	++	0
					6	+	(+)f
II					7	++	+++
					8	++	+++
XIX	Ca	Cl	Na, K, Mg	HCO_3, HPO_4, SO_4	7	++	+f
					8	+++	0
XX	Ca, Na	Cl, HCO_3	K, Mg	HPO_4, SO_4	7	+	+
					8	+++	+(+)f

* See Appendix.

† Solution II contained the following ions: Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, HPO_4^-, HCO_3^- and SO_4^{2-}.

f = movements of male gametes feeble.

[ ] = ions present in very low concentration, in acid used to adjust pH of solution.

VI. DISCUSSION

The foregoing experiments clearly show that chicken plasma or serum have no specific action in inducing the emergence of the gametocytes of *Plasmodium gallinaceum* from the corpuscles and the subsequent development of the male gametocytes, both of which can take place as well in the serum or plasma of other

animals as in those of the normal host. Moreover, the further development of the gametes in the gut of the insect is not necessarily impaired by the presence of foreign sera, since normal oocysts have been observed to develop in *Aëdes aegypti* fed upon a suspension of parasitized corpuscles in horse serum.

Apart from these experiments to test the effect of horse serum on the infectivity of *P. gallinaceum* in *A. aegypti*, the work described refers only to the emergence of the gametocytes and the exflagellation of the male gametocytes *in vitro*; the effect of immune plasma and salt solutions on fertilization and ookinete formation, and on the subsequent development of the ookinetes in the mosquito has not been studied. The present work thus has only a limited reference to that of other workers on the factors influencing the infectivity of *P. gallinaceum* for mosquitoes.

Evidence from several sources (Lumsden & Bertram, 1940; Cantrell & Jordan, 1946; Eyles, 1952*b*; Huff & Marchbank, 1955) has suggested that plasma, at the peak of parasitaemia, has a depressing effect upon the infectivity of the gametocytes in avian malaria infections, and this has tentatively been attributed to developing immunity in the vertebrate host (Huff *et al.* 1958). In the present experiments, hyperimmune chick plasma did not inhibit the emergence of the gametocytes from the erythrocytes or the development of the male gametocytes. Any inhibitory action which immune plasma may exert must therefore affect either the maturation of the female gametocytes, or some later stage in the sexual development of the parasites.

Experiments have shown that the male gametocytes can develop normally if parasitized corpuscles are suspended in isotonic solutions of certain inorganic salts. The observation that exflagellation may be unable to take place in solutions in which the gametocytes emerge from the erythrocytes suggests that the conditions required for these two stages of development may differ. In the presence of NaCl alone, few gametocytes emerged, whereas in $NaHCO_3$ alone normal numbers of gametocytes emerged, but only limited, feeble exflagellation occurred. A combination of Na, HCO_3 and Cl ions, however, enabled maximum numbers of male gametocytes to exflagellate normally and suggested that in this solution the Cl ions influenced the development of the gametocytes after they had emerged from the corpuscles. This particular combination of ions provided optimal conditions for normal exflagellation, since in such a solution the development of the gametocytes was not delayed as compared with that in whole blood.

Eyles (1952*a*) compared the numbers of ookinetes produced in *A. aegypti* fed on infected defibrinated blood and infected erythrocytes suspended in physiological saline; slightly fewer ookinetes were produced in the mosquitoes fed on saline than on blood, but Eyles considered this difference was insignificant. In the present experiments exflagellation rarely occurred in saline which suggests that, contrary to Eyles's results, few ookinetes would be formed in mosquitoes fed upon gametocytes suspended in saline. In Eyles's experiments, some time must have elapsed before the infected corpuscles were suspended in saline, whereas in the present work, the infected erythrocytes were suspended in saline immediately after being withdrawn from the bird. Since the gametocytes begin their development

very shortly after blood is exposed to air (Bishop & McConnachie, 1956), it is likely that in Eyles's experiments the gametocytes remained sufficiently long in undiluted blood for their development to proceed normally, and for this reason, the subsequent exposure to saline had no effect on fertilization and the formation of ookinetes.

VII. SUMMARY

1. Gametocytes of *Plasmodium gallinaceum* emerged from the erythrocytes and the male gametocytes exflagellated normally *in vitro*, in normal or inactivated horse serum, or in rabbit plasma.

2. Gametocytes, washed and suspended in normal horse serum, completed their sexual development and produced oocysts in mosquitoes.

3. Hyperimmune chick plasma, as compared with normal chick plasma, did not significantly affect the time of onset or the frequency of exflagellation.

4. In a solution, isotonic to bird blood and adjusted to approximately pH 8, containing NaCl, KCl, $CaCl_2$, Na_2HPO_4, $MgSO_4$, $NaHCO_3$ and Tris buffer, the *in vitro* development of the gametocytes was normal, and the numbers developing were comparable to those in horse serum, or in plasma or serum from the normal host. The development of the gametocytes was inhibited by the omission of Na or HCO_3 ions, but the omission of Mg, K, Ca, SO_4 or HPO_4 ions had no effect.

5. Further experiments showed that the gametocytes emerged from the corpuscles and the males exflagellated normally in a solution containing only Na, Cl and HCO_3 ions. Other combinations of ions were tested, but although gametocytes emerged from the corpuscles in some of these solutions, exflagellation either did not take place or did so infrequently, and the gametes produced were feeble in their movements. Within the range of inorganic salts tested, Na, HCO_3 and Cl ions constituted the minimal requirement for the normal *in vitro* development of the male gametocytes.

REFERENCES

BISHOP, A. & GILCHRIST, B. M. (1946). Experiments upon the feeding of *Aëdes aegypti* through animal membranes with a view to applying this method to the chemotherapy of malaria. *Parasitology*, **37**, 85–100.

BISHOP, A. & MCCONNACHIE, E. W. (1956). A study of the factors affecting the emergence of the gametocytes of *Plasmodium gallinaceum* from the erythrocytes and the exflagellation of the male gametocytes. *Parasitology*, **46**, 192–215.

CANTRELL, W. & JORDAN, H. B. (1946). Changes in the infectiousness of gametocytes during the course of *Plasmodium gallinaceum* infections. *J. infect. Dis.* **78**, 153–9.

CHORINE, V. (1933). Conditions qui régissent la fécondation de *Plasmodium praecox*. *Arch. Inst. Pasteur Algér.* **11**, 1–8.

EATON, M. D. (1938). The agglutination of *Plasmodium knowlesi* by immune serum. *J. exp. Med.* **67**, 857–70.

EYLES, D. E. (1952*a*). Studies on *Plasmodium gallinaceum*. II. Factors in the blood of the vertebrate host influencing mosquito infection. *Amer. J. Hyg.* **55**, 276–90.

EYLES, D. E. (1952*b*). Studies on *Plasmodium gallinaceum*. III. Factors associated with the malaria infection in the vertebrate host which influence the degree of infection in the mosquito. *Amer. J. Hyg.* **55**, 386–91.

HEILBRUNN, L. V. (1952). *An Outline of General Physiology*. Philadelphia: W. B. Saunders and Co.

HUFF, C. G. & MARCHBANK, D. F. (1955). Changes in infectiousness of malarial gametocytes. I. Patterns of oocyst production in seven host-parasite combinations. *Exp. Parasitol.* **4**, 256–70.

HUFF, C. G., MARCHBANK, D. F. & SHIROISHI, T. (1958). Changes in infectiousness of malarial gametocytes. II. Analysis of the possible causative factors. *Exp. Parasitol.* **7**, 399–417.

LUMSDEN, W. H. R. & BERTRAM, D. S. (1940). Observations on the biology of *Plasmodium gallinaceum* Brumpt, 1935, in the domestic fowl, with special reference to the production of gametocytes and their development in *Aëdes aegypti* (L). *Ann. trop. Med. Parasitol.* **34**, 135–60.

MARCHOUX, E. & CHORINE, V. (1932). La fécondation des gamètes d'hématozoaires. *Ann. Inst. Pasteur*, **49**, 75–102.

ZUCKERMAN, A. (1945). *In vitro* opsonic tests with *Plasmodium gallinaceum* and *Plasmodium lophurae*. *J. infect. Dis.* **77**, 28–59.

APPENDIX

The following solutions, all isotonic with bird blood, were made up in glass-distilled water, and the pH of each was adjusted to approximately 8

Solution I (Tris-NaCl)

NaCl 0·965 g.
Tris buffer 0·121 g. } /100 ml.
pH adjusted with N/10 HCl

Solution II

Tris-NaCl	—	(0·175 M)	100 parts
KCl	1·306 g./100 ml.	(0·175 M)	4 parts
$CaCl_2$	1·389 g./100 ml.	(0·125 M)	3 parts
Na_2HPO_4	2·48 g./100 ml.	(0·175 M)	1 part
$MgSO_4.7H_2O$	4·32 g./100 ml.	(0·175 M)	1 part
$NaHCO_3$	1·46 g./100 ml.	(0·175 M)	21 parts

pH adjusted with N/10 HCl or 10 % H_2SO_4

Solution III (solution II without $MgSO_4$)

Tris-NaCl	100 parts	Na_2HPO_4	1 part
KCl	4 parts	$NaHCO_3$	21 parts
$CaCl_2$	3 parts	pH adjusted with N/10 HCl	

Solution IV (solution II without Na_2HPO_4)

Tris-NaCl	100 parts	$MgSO_4$	1 part
KCl	4 parts	$NaHCO_3$	21 parts
$CaCl_2$	3 parts	pH adjusted with N/10 HCl	

Solution V (solution II without KCl)

Tris-NaCl	100 parts	$MgSO_4$	1 part
$CaCl_2$	3 parts	$NaHCO_3$	21 parts
Na_2HPO_4	1 part	pH adjusted with N/10 HCl	

Solution VI (solution II without $CaCl_2$)

Tris-NaCl	100 parts	$MgSO_4$	1 part
KCl	4 parts	$NaHCO_3$	21 parts
Na_2HPO_4	1 part	pH adjusted with N/10 HCl	

Solution VII (solution II without Na ions)

KCl	1·23 g.	/100 ml.	100 parts
Tris buffer	0·121 g.		
$CaCl_2$	1·389 g./100 ml.		3 parts
K_2HPO_4	3·048 g./100 ml.		1 part
$MgSO_4.7H_2O$	4·32 g./100 ml.		1 part
$KHCO_3$	1·752 g./100 ml.		21 parts

pH adjusted with N/10 HCl

Solution VIII (solution II without $NaHCO_3$)

Tris-NaCl	100 parts	Na_2HPO_4	1 part
KCl	4 parts	$MgSO_4$	1 part
$CaCl_2$	3 parts	pH adjusted with N/10 HCl	

Solution IX

Tris-NaCl	—		100 parts
$NaHCO_3$	1·36 g.	/100 ml.	21 parts
Tris-buffer	0·121 g.		

pH adjusted with N/10 HCl

Solution X

$NaHCO_3$	1·36 g.	/100 ml.
Tris buffer	0·121 g.	

pH adjusted with N/10 HCl or 10 % H_2SO_4

Solution XI

Na_2HPO_4	2·34 g.	/100 ml.
Tris buffer	0·121 g.	

pH adjusted with 10 % H_2SO_4

Solution XII

Na_2HPO_4	2·34 g.	/100 ml.	1 part
Tris buffer	0·121 g.		
$NaHCO_3$	1·36 g.	/100 ml.	21 parts
Tris buffer	0·121 g.		

pH adjusted with 10 % H_2SO_4

Solution XIII

$MgSO_4.7H_2O$	3·96 g.	/100 ml.
Tris buffer	0·121 g.	

pH adjusted with 10 % H_2SO_4

Solution XIV

$MgSO_4.7H_2O$	3·96 g.	/100 ml.	1 part
Tris buffer	0·121 g.		
$NaHCO_3$	1·36 g.	/100 ml.	21 parts
Tris buffer	0·121 g.		

pH adjusted with 10% H_2SO_4

Solution XV

KCl	1·23 g.	/100 ml.
Tris buffer	0·121 g.	

pH adjusted with N/10 HCl

Solution XVI

KCl	1·23 g.	/100 ml.	4 parts
Tris buffer	0·121 g.		
$NaHCO_3$	1·36 g.	/100 ml.	21 parts
Tris buffer	0·121 g.		

pH adjusted with N/10 HCl

Solution XVII

$KHCO_3$	1·65 g.	/100 ml.
Tris buffer	0·121 g.	

pH adjusted with N/10 HCl

Solution XVIII

KCl	1·23 g.	/100 ml.	4 parts
Tris buffer	0·121 g.		
$KHCO_3$	1·65 g.	/100 ml.	21 parts
Tris buffer	0·121 g.		

pH adjusted with N/10 HCl

Solution XIX

$CaCl_2$	1·28 g.	/100 ml.
Tris buffer	0·121 g.	

pH adjusted with N/10 HCl

Solution XX

$CaCl_2$	1·28 g.	/100 ml.	3 parts
Tris buffer	0·121 g.		
$NaHCO_3$	1·36 g.	/100 ml.	21 parts
Tris buffer	0·121 g.		

pH adjusted with N/10 HCl

Solution XXI

Tris-NaCl	—	4 parts
$KHCO_3$	1·65 g.	21 parts
Tris buffer	0·121 g.	/100 ml.

pH adjusted with N/10 HCl

Solution XXII

Tris-NaCl	—	21 parts
$KHCO_3$	1·65 g.	4 parts
Tris buffer	0·121 g.	/100 ml.

pH adjusted with N/10 HCl

Parasitology (1960), **50**, 449–452
With 1 *text-figure*
Printed in Great Britain

Duration of invasiveness of *Entamoeba histolytica* maintained *in vitro*

By PATRICIA VINCENT and R. A. NEAL
Wellcome Laboratories of Tropical Medicine, London, N.W. 1

(*Received* 6 *January* 1960)

In a previous paper, experiments were described which showed that passage in hamster liver revived the virulence of *Entamoeba histolytica* (Neal & Vincent, 1956). The present paper describes results obtained by subjecting a single strain to a series of liver passages. The results have been referred to in recent reviews (Neal, 1957; Neal, 1958).

MATERIAL AND METHODS

The strain employed (*M*) was isolated on 29 October 1947 from a case of acute relapsing amoebic dysentery (see Neal, 1951, for further details). It was grown in the inspissated horse serum slant covered with dilute egg white plus rice starch medium (*HSre* + *S*) by subculturing twice weekly. Cysts were not observed in this medium and efforts to obtain cysts *in vitro* by the technique of Dobell & Laidlaw (1926) invariably failed. The amoebae were maintained *in vitro* with a mixed unknown bacterial flora derived from the original inoculum from faeces.

The virulence of this strain has been closely watched because it was used for routine chemotherapy experiments, for which batches of 50 to 100 rats were inoculated each week. Five to ten inoculated rats were left untreated and acted as controls for the infectivity and invasiveness of the amoebae. The data thus accumulated over 12 years are too vast to be presented in detail, and they have been summarized by making an estimate of the infectivity and invasiveness every 6 months, usually in March to April and September to October of each year. During each of these periods, the averages of infectivity and invasiveness were calculated from four to six consecutive experiments, each experiment being composed of six to ten untreated rats inoculated with amoebae. The infectivity was expressed as the percentage of rats infected with *E. histolytica* and the invasiveness by the average degree of ulceration calculated from the caecal scores of all control rats in each experiment (Neal, 1951). It should be noted that this is an important difference in description of results. In this paper where a loss of invasiveness is being described, the average caecal score of all inoculated but untreated rats is calculated, whereas in previous papers the average caecal score of infected rats only was reported. The amount of variation of infectivity and invasiveness between experiments at each 6-monthly period is expressed as the standard error, represented in Fig. 1 by vertical lines through each point in the graphs.

Rats aged 3–4 weeks were inoculated by intracaecal injection of about 50×10^3 amoebae in 0·3–0·4 ml. of inoculum taken from 48 hr. cultures. The animals were fed on a dry mash of constant composition. They were killed 7 days after inoculation.

Their caeca were examined macroscopically for ulceration and their intestinal contents microscopically for amoebae. If no amoebae were observed, cultures were prepared in *HSre+S* medium and examined after 48 hr. incubation at 37° C.

When the invasiveness had dropped to an average degree of ulceration which scored less than 3·0, a series of liver passages was made. An average of less than 3·0 was chosen since a rat's caecum with minimal but definite amoebic ulceration is given a score of 3. As described earlier (Neal & Vincent, 1956) the technique of Reinertson & Thompson (1951) was used. About 25×10^3 amoebae were inoculated in 0·1 ml. of suspension beneath the hepatic capsule of the golden hamster (*Mesocricetus auratus*), a group of three to six animals being inoculated at each passage. The hamsters were killed 3–4 days after inoculation and a portion of the amoebic lesion inoculated into *HSre+S* medium. After growth for 2–3 days the amoebae were inoculated into a further group of hamsters. When amoebic lesions were found in more than one animal at autopsy, the amoebae were recovered from all animals and the cultures which grew best were used for inoculation into rats or hamsters. If no difference in growth rate was observed in the cultures made from the hamsters, the amoebae from all animals of the same group were pooled before inoculation into another group of hamsters.

RESULTS AND DISCUSSION

The summarized results collected from 1947 to 1959 are shown in Fig. 1. These show that the infectivity and invasiveness of the original strain *M* remained constant for 290 weeks and then lost its infectivity and invasiveness over a further period of about 24 weeks. The attenuated *M* strain was then given the first series of five liver passages. This revived the virulence, although to a slightly lower level than that of the original strain. Three strains were isolated from hamsters in the fifth passage of this series, *MLVa*, *MLVb* and *MLVc*.

The virulence of two of these, *MLVb* and *MLVc*, was studied further. Virulence was lost after 62 and 183 weeks of cultivation *in vitro* respectively. Strain *MLVc* was given a second series of four liver passages in hamsters. Again, the amoebae isolated *in vitro* from the fourth passage (*MALIV*) were virulent, but 69 weeks later virulence had again decreased. A third series of six liver passages again increased the virulence, but the virulence of the re-isolated amoebae (strain *MHVI*) lasted for only 45 weeks.

These results show that the loss of virulence during cultivation *in vitro* is a gradual process. It may commence, as in the case of *M* strain, abruptly and decrease over a relatively short time, or attenuation may proceed gradually over a year or more, as observed during the study of the strains invigorated by liver passage. The average degree of ulceration dropped to below 3·0; the infectivity remained above 65 % in all cases except for the original *M* strain in which the infectivity decreased to 48 %. From this it may be concluded that the amoebae remain infective to young rats, but partially or wholly lose their capacity to invade the caecal mucosa. The slow loss of virulence of *E. histolytica* has been previously described in detail by Thompson, McCarthy & Reinertson (1954) and our results agree with theirs.

Although our observations show that the virulence may be successfully revived by liver passage, the duration of the experimentally acquired virulence is variable (Table 1). The virulence of three strains isolated *in vitro* from different hamsters in the same group from the first series of liver passages lasted for less than 40 weeks (strain *MLVa*, see Neal & Vincent, 1956), 62 weeks (strain *MLVb*) and 183 weeks (strain *MLVc*) respectively. Also, strains isolated from hamster liver lesions after the second and third series of liver passages remained virulent for 69 and 45 weeks

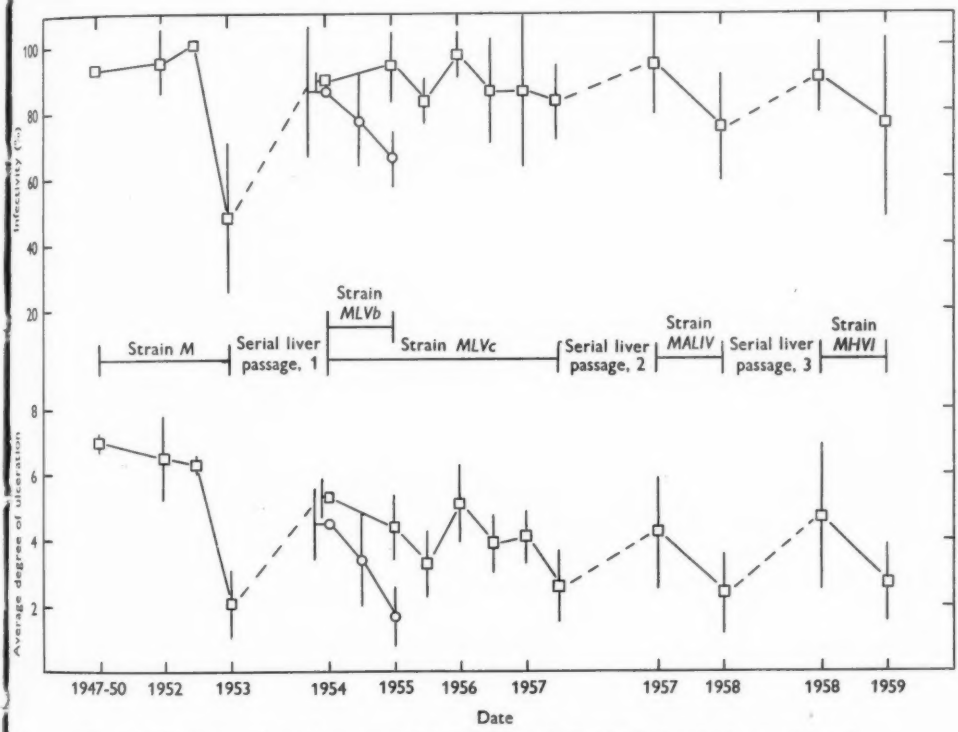


Fig. 1. The loss of invasiveness and infectivity of *E. histolytica* during prolonged cultivation *in vitro* and their restoration by liver passage. The points for 1947 to 1950 were taken from Neal (1951). The vertical lines through each point represents the standard error.

Table 1. *Duration of virulence after restoration by successive series of liver passages*

Series of passages	No. of liver passages in each series	Name of strain	Duration of virulence (weeks)
—	—	*M*	290
1	5	*MLVb*	62
1	5	*MLVc*	183
2	4	*MALIV*	69
3	6	*MHVI*	45

respectively. If the strains *M*, *MLVc*, *MALIV* and *MHVI* are considered as a series, it is seen that there is a progressive shortening of the duration of virulence after each series of liver passages (290, 183, 69 and 45 weeks respectively). This may not be a valid conclusion on the grounds that the duration of virulence of one strain from the first passage (*MLVb*) was shorter than that from the second passage (*MALIV*) while another strain from the first passage (*MLVA* less than 40 weeks; Neal & Vincent, 1956) retained its virulence for a shorter time that did a strain from the third passage (*MHVI*). However, amoebae isolated *in vitro* after the second and third series of passages were in fact derived from pooled amoebae from all hamsters of each passage, and the variation of virulence was therefore minimized.

Although the amoebae used in these experiments are descendants of those isolated from the human host in 1947, the bacterial flora may have changed during the subcultivation and during liver passage. The present experiments throw no further light on the role of the concomitant bacteria. However, the results of earlier work (Neal, 1956) showed that the virulence of an attenuated strain of *E. histolytica* could not be increased by the addition of bacteria taken from the same strain after its virulence had been increased by liver passage.

SUMMARY

The infectivity and invasiveness of a single strain of *Entamoeba histolytica* to young rats has been studied over a period of 12 years. The virulence was slowly lost over a period of months or years of cultivation *in vitro*. After four to six liver passages, virulence was again established and again declined in culture. This was repeated on three occasions. Each time the strain lost virulence, the capacity to invade the mucosa was lost before the amoebae became non-infective to young rats. The duration of experimentally acquired virulence was progressively reduced after each succeeding series of liver passages.

REFERENCES

DOBELL, C. & LAIDLAW, P. P. (1926). On the cultivation of *Entamoeba histolytica* and some other entozoic amoebae. *Parasitology*, **18**, 283.

NEAL, R. A. (1951). Some observations on the variation of virulence and response to chemotherapy of strains of *Entamoeba histolytica* in rats. *Trans. R. Soc. trop. Med. Hyg.* **44**, 439.

NEAL, R. A. (1956). Strain variation in *Entamoeba histolytica*. III. The influence of the bacterial flora on the virulence to rats. *Parasitology*, **46**, 183.

NEAL, R. A. (1957). Virulence in *Entamoeba histolytica*. *Trans. R. Soc. trop. Med. Hyg.*, **51**, 313.

NEAL, R. A. (1958). The pathogenicity of *Entamoeba histolytica*. *Proc. 6th Int. Congr. trop. Med. Malaria* (in the Press).

NEAL, R. A. & VINCENT, P. (1956). Strain variation in *Entamoeba histolytica*. II. The effect of serial liver passage on the virulence. *Parasitology*, **46**, 173.

REINERTSON, J. W. & THOMPSON, P. E. (1951). Experimental amoebic hepatitis in hamsters. *Proc. Soc. exp. Biol., N.Y.*, **76**, 518.

THOMPSON, P. E., MCCARTHY, D. & REINERTSON, J. W. (1954). Observations on the virulence of *Entamoeba histolytica* during prolonged subcultivation. *Amer. J. Hyg.* **59**, 249.

Parasitology (1960), **50**, 453–468
With 1 plate and 5 text-figures
Printed in Great Britain

The functional anatomy of the cysticercoid of *Choanotaenia crassiscolex* (Linstow 1890) (Dilepididae) from the digestive gland of *Oxychilus cellarius* (Müll) (Stylommatophora), with some observations on developmental stages

By DILYS RAWSON and JOYCE E. RIGBY
Department of Biology, Queen Elizabeth College (University of London)

(*Received* 20 *January* 1960)

During the course of work by one of the authors, involving some 600 specimens of *Oxychilus cellarius*, it was found that approximately 3% of the specimens were infected with the cysticercoid of *Choanotaenia crassiscolex* (Pl. 1, fig. 1). The snails were collected at various times of the year in a disused chalk quarry in Surrey, infected specimens being recorded in May, June and October.

Identification of the parasites presented some difficulty and so far we have not had an opportunity of following the life cycle in an experimental definitive host. The material has, however, been examined by Mr S. Prudhoe of the British Museum of Natural History, South Kensington, and compared with his specimens of the adult *Choanotaenia crassiscolex* from *Sorex araneus* L. (the common shrew). The material was also seen by Professor J. G. Baer, University of Neuchâtel, who pointed out its similarity to *Cysticercus* E of Harper (1930) from the same intermediate host and listed (Joyeaux & Baer, 1936) under unknown life cycles. We have since compared our measurements with those of Harper and found that they are almost identical except for the number of rostellar hooks. This, however, is an error likely to arise unless the hooks are separated and there is no evidence that Harper did this. Since we started this work, a paper by Kisielewska (1958) has come to our notice, giving a very brief description of a cysticercoid identified as *Choanotaenia crassiscolex* found in various Stylommatophora in the Bialowieza National Park, Poland. In this same area the author found what is believed to be the adult form in various insectivores, but no infection experiments were carried out. There is some agreement between her measurements and ours, and her drawings show close similarity to our material. We give below a more detailed account of the anatomy of our cysticercoids, since no such description of any cysticercoid could be found in existing literature.

TECHNIQUE

As far as possible the living as well as preserved material was studied. Measurements were made from preserved material. Living material was observed and drawn in invertebrate saline. Helly's fluid or Bouin were used as fixatives and some of the worms were sectioned *in situ* and others after dissection. Sections were cut at $7\frac{1}{2}$ and 6μ in transverse and longitudinal planes and stained in Heidenhain's haematoxylin and Southgate's mucicarmine, the latter being a histochemical

stain for mucopolysaccharides. Preserved material, after removal from the host, was mounted in Berlese fluid with gentle heat* for phase-contrast examination of separated hooks, cuticle and calcareous bodies. The drawings of hooks and cuticular spines were made with the help of phase-contrast photomicrographs taken under $\frac{1}{12}$ in. oil immersion objective. Whole mounts were stained in Mayer's Paracarmine.

I. EXTERNAL MORPHOLOGY OF CYSTICERCOIDS

In the host the worms are found projecting from the surface of the anterior digestive gland close to the intestine and never adjacent to the outer branches of the digestive gland (Pl. 1, fig. 1). They are surrounded by a cyst wall, probably an adventitious structure, formed from host tissue. The number of cysts in any single infection varied between one and fifty. Where more than one cyst occurred adjacently to each other, their outer cyst walls were fused. Between the worm and the cyst wall is a material which reacts positively to mucicarmine indicating the presence of mucopolysaccharides. This is the 'gelatinous' zone referred to by previous writers. The cysts are remarkably constant in size especially at the most advanced stage. At this stage they measure 0·4 mm. long × 0·35 mm. wide × 0·23 mm. in depth, and so may be described as having a slightly flattened oval shape. The youngest cysts we encountered were only slightly smaller and measured 0·35 mm. in length with other measurements in proportion. Contrary to expectation, the larger infections showed at least three stages in development, though invariably most of the specimens recovered were in the condition shown in Pl. 1, fig. 6, and in Text-fig. 2*a*. In only two cases was the earliest stage (Text-fig. 1) encountered. This stage we have previously described briefly, without identification (Rigby & Rawson, 1958), and we called it the pre-cystic stage. The suitability of this term is discussed later.

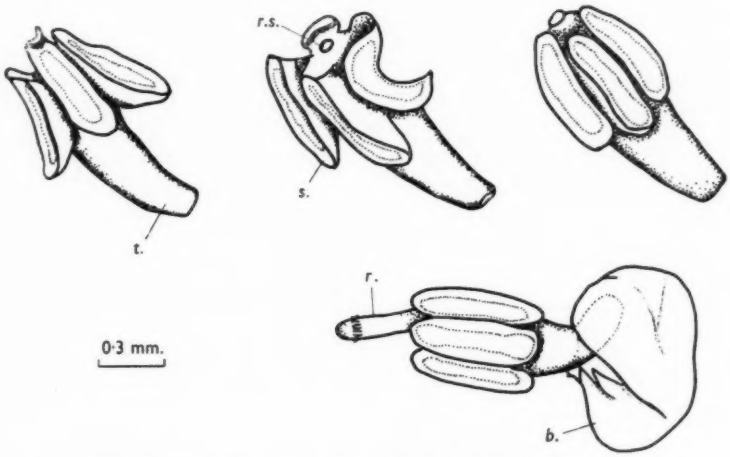
(*a*) *1st-stage larva* (Text-fig. 1). Throughout the material we found no larva earlier than this stage. On puncturing the cyst in saline the larva emerged and became quite actively mobile exhibiting the movements shown in Text-fig. 1. This larva has a fully formed scolex with a rostellum and four very long pedunculate and lobed adhesive organs, two dorsal and two ventral, with very freely mobile margins. At this stage the adhesive organs measure 0·22–0·25 mm. long with their combined widths covering most of the scolex. The margins of the adhesive organs expand anteriorly and posteriorly into well-marked lobes which protrude away from the scolex and overhang the cavity. The four adhesive organs tend to move in diagonal pairs and can move independently, their movements being facilitated by their pedunculate shape. There is a well developed, very long rostellum at this stage, armed distally with a double circle of characteristic dilepid hooks. In the saline medium the rostellum was seen to protrude and retract from time to time (Text-fig. 1).

Behind the scolex the 1st-stage larva bears a blunt rather bulbous elongated tail similar to that of most plerocercoids. The 'tail' varies in length and width but is generally at least equivalent in length to the whole scolex 0·25 mm. We

* This technique was suggested to us by Professor J. G. Baer.

could find no trace of hexacanth hooks on this tail, which has a thin coat of superficial muscles beneath the cuticle. The remainder consists of loosely packed parenchymatous cells around a central cavity filled with fluid which forms an excretory bladder opening posteriorly by an excretory pore. There is no sharp demarcation between scolex and 'tail' at this stage, in fact some specimens show a widening of the 'tail' just behind the scolex where in slightly older specimens the 'bladder' of the cysticercoid arises.

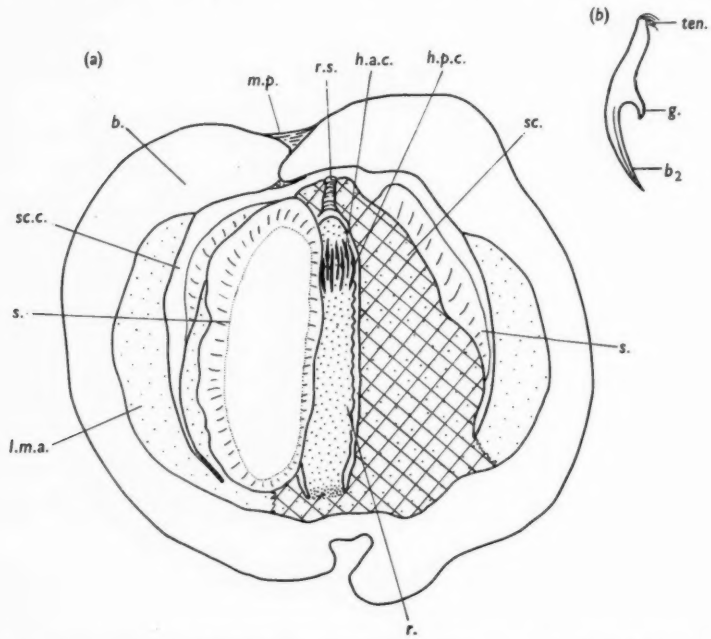
(*b*) *2nd-stage larva* (Pl. 1, figs. 3, 4). Still enclosed within the cyst, the tail region now becomes sharply differentiated into an anterior region which gives rise to the bladder and a narrow 'tail' 0·36 mm. long. The cyst wall imposes a change of shape by pressure so that most of the fluid of the 'tail' is pushed into the bladder.



Text-fig. 1. 1st-stage larva. Attitudes adopted during life.

The bladder rapidly extends forward on all sides to enclose the scolex which therefore now lies in a new 'scolex cavity'. Immediately anterior to the scolex the rim of the bladder forms a circular aperture leading into the 'scolex cavity'. This aperture is sealed and there is never any infiltration into the 'scolex cavity' of mucicarmine-positive material from the mucopolysaccharide zone surrounding the worm. The inner and outer walls of the bladder around the scolex remain separated by the turgidity of the bladder fluid believed by Smyth (1947) to be a transudate of host serum. Meanwhile, the slender 'tail' becomes pointed distally and occupies the remaining space in the cyst, becoming flattened and wrapped around one side of the bladder (Pl. 1, fig. 4). The four adhesive organs alter their shape slightly and their margins follow the oval outline of the cyst wall. The scolex thus lies in an invagination of the bladder and a cercocystis (with a 'tail' enclosed in the cyst) type of cysticercoid is formed, of the kind in which the differentiation of the scolex precedes the formation and invagination of the bladder. Within the cyst the worm makes limited movements but the rostellum always remains retracted.

(*c*) *3rd-stage larva* (Text-fig. 2*a*; Pl. 1, figs. 5, 6). At this stage the appearance of the scolex and bladder is the same as in the preceding stage but the 'tail' has become further constricted from the bladder and diminished in size to a small stump (Pl. 1, fig. 5). Some specimens show no 'tail' at all but only an indentation of the bladder wall at the point of the 'tail' constriction (Text-fig. 2). This has led many writers to suppose that the tail drops off. In fact, the present series of photographs show that it is much more likely that the tail is gradually incorporated into the bladder and that the constriction, apparently stationary, relative to the scolex and the cyst wall is really moving slowly down the 'tail' as the latter is



Text-fig. 2(*a*). 3rd-stage larva without 'tail', removed from cyst. Half of the bladder wall, one sucker and the scolex and rostellar capsule beneath it (cross-hatched area) have been removed to expose the rostellar sac and rostellum. (*b*) Hook.

drawn into the bladder. This would account in part for the noticeable increase in size of the bladder at this stage. The whole cyst and the worm look perceptibly larger now and reach the maximum size noted earlier. A detailed account of the structure of the worm at this stage follows later, and measurements of cysts, scolex and suckers and width of outer zone are stated for this stage in Table 1. Comparable measurements for this worm given by Harper, Baer and Kisielewska, and also the measurements of Mr Prudhoe's adult specimens, are included in Table 1.

(*d*) *4th-stage larva* (Pl. 1, figs. 7, 8). The photographs show the fully developed cysticercoid at its maximum size, and measurements and drawings of hooks are given for this stage, measurements of hooks being taken in a straight line from the tip of the blade to the base of the handle. The scolex is shown emerging from

the 'scolex cavity' in the bladder (Pl. 1, fig. 7) and also fully protruded (Pl. 1, fig. 8). Owing to the shape of the cyst and also the musculature of the worm, referred to later, the scolex slips to one side of the slightly flattened cyst, giving the curled appearance characteristic of *Choanotaenia infundibulum* drawn by Reid & Ackert (1937). The extended position shown in Pl. 1, fig. 8 was achieved by pressure and rolling of the freshly mounted specimen. The latter figure shows the extension that has taken place at the base of the scolex which must assist the emergence of the scolex. Consequently the bladder slips backwards breaking the

Table 1. *Measurements in mm. of* Choanotaenia crassiscolex *from five records*

	Joyeaux & Baer (adults)	Harper (cysticercoids)	Kisielewska (*a*) late cysticercoids (*b*) adults	Rawson & Rigby (*a*) cysticercoids (*b*) N.H. Museum adults
Size of cyst	—	0·5 × 0·48	—	0·4 × 0·35
Width of muco-polysaccharide zone	—	0·056	—	0·03
Width of scolex	0·5–0·75	—	(*a*) 0·45–0·6 (*b*) 0·64–0·72	(*a*) 0·2 (*b*) 0·48
Length of suckers	0·3–0·4	0·2	(*a*) 0·160–0·37 (*b*) 0·42–0·44	(*a*) 0·2 (*b*) 0·45
Width of suckers	0·02–0·04	0·08	—	(*a*) 0·1 (*b*) 0·2
No. of hooks	16–18	15	(*a*) 18 (*b*) 18	(*a*) 20 (*b*) ?
Size of hooks	0·052–0·055	—	(*a*) 0·046–0·056 (*v*) 0·048–0·056	(*a*) 0·05 (*b*) 0·06

plug at the entrance to the 'scolex cavity', and the bladder thus becomes posterior in position and nearly circular in shape. Meanwhile the adhesive organs lose their lobes and peduncles as the scolex expands though they remain long and oval in outline.

II. FUNCTIONAL ANATOMY OF THE 3RD-STAGE LARVA

(Text-figs. 2*a*, 3, 4, 5; Pl. 1, figs. 5, 6)

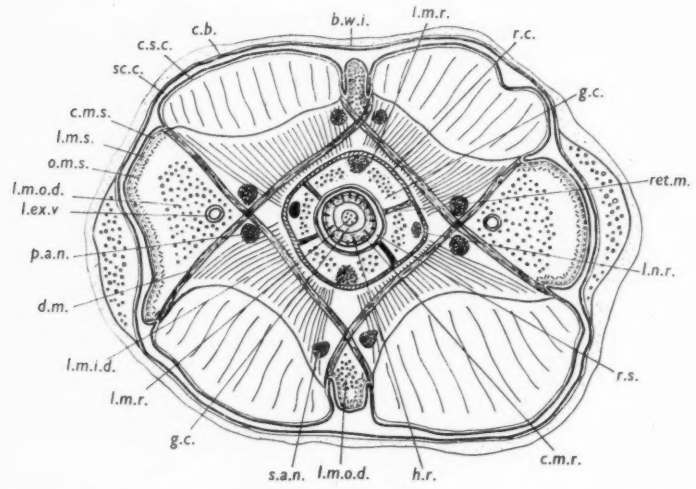
A reconstruction of the scolex musculature and some nerves and excretory vessels have been made from macerations and sections of worms with the rostellum retracted. The entire worm is covered on the outside by a cuticle which is thin and smooth over the bladder, but thickened and serrated or spinose (backwardly directed) over the entire scolex including the lining of the cavity of the rostellar sac. The spines are largest on the edges or rims of the adhesive organs and seemingly absent from the anterior part of the rostellum. Beneath the cuticle are circular and longitudinal superficial muscles followed by oblique muscles in the scolex region. Deep musculature is developed only in the scolex, the bladder being filled with fluid, apart from the large parenchyma cells containing calcareous bodies which are most numerous at the base of the scolex. The limited movements normal

at this stage, must be brought about largely by the superficial muscles of the bladder exerting a pressure on the fluid interior, so facilitating the circulation of the fluid to all areas.

(1) *Musculature and glands*

(*a*) *The rostellar apparatus*

The rostellar sac penetrates the length of the entire scolex, and is therefore about equivalent in length (0·24 mm.) to the very long adhesive organs. It is formed by an inpushing of the scolex 0·04 mm. in diameter around the base of the rostellum, the lining of its cavity being continuous with the body layers of the scolex and rostellum. These layers comprise a cuticle, strong superficial circular, superficial longitudinal and also well-developed deep longitudinal muscles. The latter form a cylindrical column attached at the scolex sphincter (Text-fig. 4) and

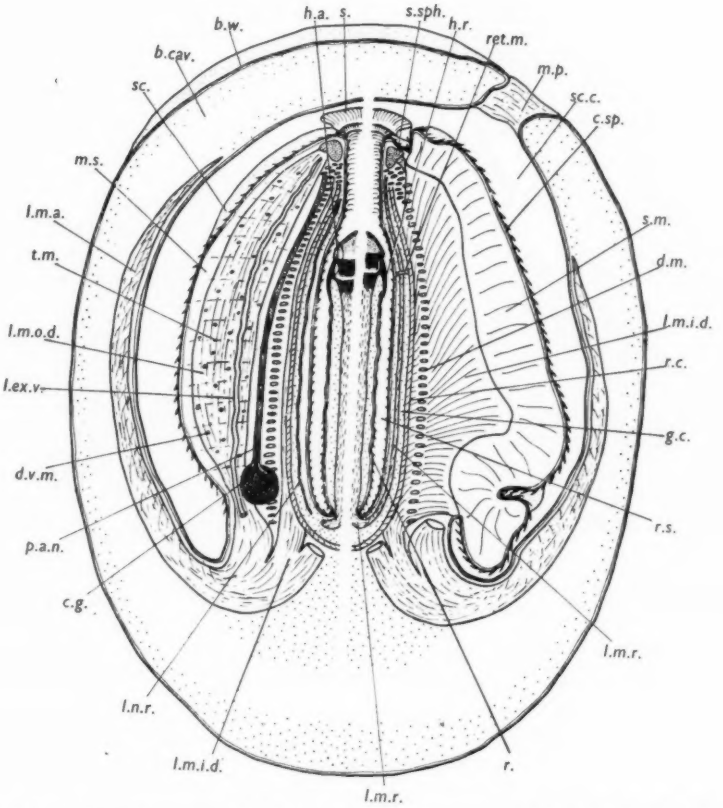


Text-fig. 3. Diagrammatic representation of musculature of 3rd-stage larva, based on transverse sections. The outside of the bladder and transverse and dorso-ventral muscles are omitted.

running to the tip of the rostellum. This cylinder is best developed in the median dorsal and median ventral lines of the rostellar sac (Text-fig. 3). The lining of the posterior part of the rostellar sac forms the posterior half of the protruded rostellum and is invaginated again when the anterior part of the rostellum is retracted. The rostellar apparatus is limited by the rostellar capsule (Text-figs. 3, 4) formed from deep diagonal muscles which have dipped inwards posterior to the scolex sphincter at the base of the rostellum. These capsule muscles also enclose a fluid-filled space between themselves and the rostellum amongst which are many glandular cells, and like those in the uninvaginated half of the rostellum possibly secrete a lubricating substance into the rostellar sac to help movements of the rostellum. The walls of the rostellar sac and the capsule are joined anteriorly by two lateral pairs of stout retractor muscles (Text-figs. 3, 4 *ret.m.*) necessary for retraction of

the rostellum. Structures similar to these in *Dreparidotaenia* are regarded by Benham (1900) as septa. The rostellar capsule does not evaginate, and is probably responsible in the main for the protrusion of the rostellum, helped by the intrinsic rostellar musculature and the muscles of the scolex.

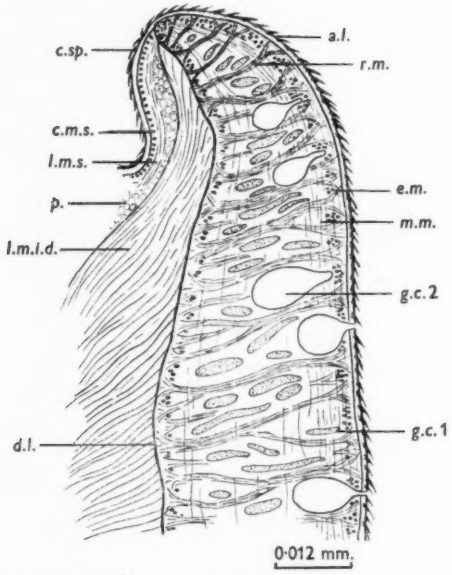
To bring about protrusion of the rostellum, the diagonal muscles of the capsule must first contract and as they are oblique, will not alter its shape in doing so, and



Text-fig. 4. Reconstruction of 3rd-stage larva, representing a longitudinal sagittal half of the larva with the sucker removed from the left side. The right half is cut in a diagonal plane, the left half in the lateral plane. Superficial musculature is omitted and transverse and dorso-ventral muscles are shown only on the left side.

the hydrostatic pressure of the fluid between the capsule and rostellum will keep the capsule turgid. This movement is presumably accompanied by contraction of the superficial circular and relaxation of the superficial longitudinal muscles of the rostellum, causing a general elongation of the rostellum with the hooks pressed on to its surface for minimum resistance to forward movement. In addition it would be necessary for the deep longitudinal muscles of the rostellum attached to the scolex tip, to be strongly contracted, while the retractor muscles connecting

the rostellar sac and capsule walls anteriorly, and the circular muscles of the 'scolex-sphincter' (Text-fig. 4) must simultaneously relax. The initial contraction of the capsule muscles is probably assisted by scolex musculature, viz. the contraction of deep diagonal muscles (Text-fig. 3) and the corresponding relaxation of inner deep longitudinal muscles to the suckers, described later. The turgidity of the expanded rostellum must be maintained by hydrostatic pressure as suggested by Benham (1900).



Text-fig. 5. Longitudinal section of portion of sucker. The apparent anastomosis of radial muscles is caused by pressure from gland cells.

No satisfactory explanation has yet been given of how the rostellum is retracted. It would seem to involve initially a contraction of the retractor muscles between the capsule wall and the rostellar sac, the latter now forming the posterior part of the evaginated rostellum. This movement would need to be accompanied by a relaxation of the rostellar deep longitudinal muscles. Simultaneous contraction of rostellar superficial longitudinal muscles and corresponding relaxation of rostellar superficial circular muscles would cause the necessary shortening of the rostellum. The scolex muscles would need to relax completely except for the circular sphincter muscles which will finally enclose the retracted rostellum in the rostellar sac once again. Retraction of the rostellum would probably cause a movement of fluid from the rostellum and the space between it and the capsule wall through the meshwork of oblique muscles of the capsule wall to the scolex. The length of the protrusible anterior half of the rostellum, relative to the invaginated half now forming the rostellar sac wall, is determined by the position of the retractor muscles (Text-figs. 3, 4). Such muscles have not previously been identified in any rostellum. Benham (1900), who found what he describes as an extrinsic muscula-

ture of the rostellar capsule in *Dreparidotaenia*, did not suggest how the rostellum was invaginated.

Near the tip of the rostellum the superficial circular muscles become thickened in four circular bands into which are sunk the hook pockets of the double ring of hooks. Both the handles and guards of each hook are in a pocket, those of the handles set into the two more anterior, and those of the guards into the two posterior circular muscle bands. Inside each pocket the hook has a tendon (Text-fig. 2*b*) to which are attached oblique muscles and deep longitudinal muscles from the central column in the rostellum. By contraction the circular muscle bands press the hooks on to the rostellar surface giving a streamlined outline for forward or backward movements of the rostellum. The longitudinal muscles of the hooks by contraction cause elevation of the backwardly directed blades for acquiring a grip into the intestinal mucosa, while the oblique muscles allow for bodily movements of the worm to continue after the hooks are embedded in the host tissue, so causing the minimum of tearing.

The extreme tip of the rostellum in front of the hooks is a muscular cushion with its own intrinsic musculature resembling a weak sucker. It is provided with radial, longitudinal and circular or equatorial muscles.

(*b*) *The four adhesive organs* (Text-figs. 2*a*, 3, 4, 5)

The four muscular adhesive organs are separated internally from the general scolex tissue by a definite delimiting layer. Their intrinsic musculature consists of equatorial (= circular), radial and meridional (= longitudinal) muscle fibres (Text-fig. 5). The equatorial muscles continue right around the adhesive organ beneath the outer cuticle and reach their strongest development in the anterior and posterior lobes. Radial muscles are strongly developed, especially in the lobes, but meridional (longitudinal) muscles are exceptionally weak and occur mostly near the outer edges. Interspersed amongst this musculature are numerous gland cells, best developed in the lobes. Some of these in which the nuclei take up haematoxylin (Text-fig. 5, *g.c.* 1), probably secrete an adhesive material, the remainder being very large cells like vertebrate goblet cells and apparently completely colourless with mucicarmine (Text-fig. 5, *g.c.* 2).

The latter are best developed in the earliest stage of the cysticercoid and it is reasonable to suppose that they secrete the substance filling the 'scolex cavity', which would otherwise collapse. This substance must be sufficiently dense to form a barrier to the infiltration of host muco-polysaccharides which would otherwise take place and in fact forms the visible plug at the rim of the bladder anterior to the scolex. The completely empty appearance in the sections, both of the large gland cells of the adhesive organ and the contents of the scolex cavity, would lead one to suspect that this secretion might contain quantities of fatty acids which would be expected to be capable of forming the kind of barrier necessary. The complete absence of any mucicarmine-positive material in the worm, and the fact that the mollusc very readily forms mucicarmine-positive muco-polysaccharides, suggests that everything external to the worm except the contents of the scolex cavity, is formed from host tissue. The cyst wall is therefore likely to be the

solidified outer portion of the muco-polysaccharide zone in the production of which the host amoebocytes, seen in numbers around the cysts (Pl. 1, fig. 2), are likely to have played some part.

(*c*) *Scolex musculature* (Text-figs. 3, 4)

The superficial musculature of the scolex has already been mentioned. The deep muscles are of four kinds—diagonal, longitudinal, dorso-ventral and transverse. Except for half of the longitudinal muscles the deep musculature is associated with the adhesive organs and forms their extrinsic musculature whereby they acquire elaborate independent movement.

(i) *Deep longitudinal muscles.* These are attached proximally in the anterior wall of the bladder in a lateral position (l.m.a. Text-figs. 2*a*, 4) in a wide band measuring 0·14 mm. in transverse and longitudinal planes and 0·03 mm. deep in sagittal section. Where these deep muscles are attached to the inverted bladder outside the scolex base the superficial muscles of the bladder are specially well developed. The deep longitudinal muscles pass into the scolex in two broad lateral bands to supply the right and left halves of the scolex, respectively. At the base of the scolex each band divides into two, forming the inner and outer deep longitudinal muscles of each side. Each of these groups again divides, forming a total of eight bands, the four internal ones supplying the adhesive organs and the four outer ones the scolex between the adhesive organs. The outer layer passes right through the scolex to its sphincter, converging to form a cylindrical column behind the sphincter of superficial circular muscles around the orifice of the rostellar cavity. Thus they make up much of the structure of those parts of the scolex forming four lobes, two large lateral and two small dorsal and ventral, between the adhesive organs (Text-fig. 3). The deep longitudinal muscles to the rostellum referred to earlier belong to this group of outer muscles, but are inserted at the level of the sphincter making possible the independent movement of the rostellum. The four inner longitudinal muscle bands at the base of the scolex form a cylinder immediately surrounding the rostellar apparatus and pass forwards first longitudinally around it and then obliquely in four groups to the extensive inner margins of the adhesive organs. Each of these four groups therefore spread out in a wide fan in both transverse and longitudinal planes forming an important part of the extrinsic musculature. Along their course these very long muscles are held in place by the deep diagonal muscles described below, and they obviously control the size of the vacuum created during the attachment of the adhesive organs.

(ii) *Deep diagonal muscles.* These are the most strongly developed muscles in the scolex and like the inner deep longitudinal bands form part of the extrinsic musculature of the adhesive organs. They cross the scolex as four diagonal sheets throughout its length enclosing the rostellar apparatus centrally and connecting distally the lateral borders of the adhesive organs, each pair of sheets of diagonal muscles connecting a diagonal pair of adhesive organs. The diagonal muscles cross through each other dorsally, ventrally and laterally outside the rostellar apparatus (Text-fig. 3). As extrinsic muscles they obviously control the edges of the adhesive organs, and are best developed at the anterior end of the scolex in the region of the

anterior lobes and would account for the pronounced differentiation of these lobes as well as the tendency towards a synchronization of diagonal pairs during the movements described earlier. These diagonal muscle sheets also provide the right support for the extrinsic longitudinal muscles enabling the latter to operate longitudinally, obliquely and even transversely between the adhesive organs and the centre of the scolex. Probably also the combined action of the diagonal muscles assist the contraction of the oblique muscles of the rostellar capsule wall in initiating the protrusion of the rostellum.

(iii) *Transverse and dorso-ventral muscles.* These muscles are only weakly developed compared with the scolex muscles described above. They do, however, provide accessory movement by connecting adjacent adhesive organs in transverse and dorso-ventral planes. Of the two groups, the dorso-ventral muscles are much stronger and cause the slight dorso-ventral flattening of the scolex.

(2) *Excretory system*

This system is little differentiated at this stage, presumably on account of the small size of the worm and the comparatively large volume of fluid in the bladder, conditions favouring easy and rapid diffusion. One pair of lateral longitudinal excretory vessels 0·005 mm. in diameter were detected, running through the scolex external to the anterior lateral nerve cords (Text-figs. 3, 4).

(3) *Nervous system* (Text-figs. 3, 4)

A pair of cerebral ganglia 0·015 mm. in diameter are situated laterally, one on either side of the posterior end of the rostellar apparatus. From these there arise at this stage only the anterior nerves; the posterior nerves are not differentiated. The anterior nerves consist of four longitudinal pairs comprising a pair of laterals on each side, a dorsal pair and a ventral pair, all running forwards outside and parallel to the rostellar apparatus. Each cerebral ganglion therefore connects with four longitudinal nerves, a lateral pair and a single dorsal and single ventral nerve. Of these the two pairs of laterals are the largest (0·006 mm. wide) and may be termed the primary lateral anterior longitudinal nerves, situated immediately outside the rostellar apparatus and in the angle formed by the lateral crossing of the deep diagonal muscles (Text-fig. 3). The smaller dorsal and ventral pairs comprising the four secondary anterior longitudinal nerves are not so close to the rostellar apparatus and each runs immediately beneath the inner median border of one of the four adhesive organs. A total of eight anterior longitudinal nerves therefore form a hexagon around the rostellar apparatus in much the same fashion as that described by Cohn (1898) in *Cysticercus tenuicollis* (= *Taenia hydatigena* Pallas, 1766). Although the finer branches of the nervous system could not be made out with certainty, it was apparent that the four secondary anterior longitudinal nerves supply the four adhesive organs, and the primary lateral anterior longitudinal nerves supply the rostellar capsule. A single pair of lateral nerves could be detected in the posterior invaginated part of the rostellum; these probably represent an anterior extension of the primary lateral anterior longitudinal nerves supplying the rostellum itself.

III. DISCUSSION

Published work on cysticercoids is in general scanty, frequently inaccurate or incomplete, and the literature is often confusing. Cysticercoids occur in a wide range of hosts and of positions. Most of those described have been found in small arthropods, usually in the haemocoel, and only few have been described from molluscs. The descriptions are, in the main, of broad morphology with very little reference to micro-ecology and little of the work includes photographs. Recently Schiller (1959), working on large numbers of cysticercoids of *Hymenolepis nana*, has published interesting data with photographs and has shown that variability within the species in one host type is only marked in relation to the extent of the development of the 'tail'. Until more work of this kind and more careful anatomical studies are made, it is premature to attempt an interpretation of the relationship between the larval stages of individual species and those of the Cyclophyllidea in general. No review of cestode larvae can therefore be given and the reader is referred to Furhman (1928) and Hyman (1951) for terminology and further references.

Nevertheless, we think it is necessary to direct attention to two features of interest which have emerged in the present study of *Choanotaenia crassiscolex*. These are, first, the peculiar form of the adhesive organs, and secondly, the debatable significance of the early 'tailed' larva (Text-fig. 1). The foregoing description shows that the adhesive organs in their early stages bear a remarkable resemblance to tetraphyllidian bothridia, with their very long oval and pedunculate shape, weak longitudinal musculature, and also the occurrence of distinct anterior and posterior lobes. Whether or not we can regard the present form as anything more than a case of convergence we do not know. The presence of such organs coupled with the mobile 'tail' at the 1st stage led us to regard this larva as something equivalent to a normal plerocercoid stage (i.e. post-hexacanth and pre-cystic), a stage considered to be 'absent or only slightly indicated' in cyclophyllidean development (Hyman, 1951).

Amongst the Cyclophyllidea a procercoid stage has been described for *Hymenolopis diminuta* by Joyeaux (1922). Plerocercoid-like stages, called di- or tetrathyridia, with invaginated scolices, occur in some Mesocestoididae and Nematotaeniidea and as an exception in the Dilepididae (in the genus *Gryporhynchus* Nordmann) according to Furhman (1928). Similar 'plerocercoids' occur in some Proteocephaloidea, an order with both tetraphyllidean and cyclophyllidean features. It is presumed that such larvae become cysticerci, so that it appears that a precysticercoid plerocercoid is not so far reported. If such a stage ever occurs it would be expected in the type of life history described here for *Choanotaenia crassiscolex**. The fact that we did not find any stage earlier than we report in all our material, though negative evidence, supports this kind of interpretation

* The fact that *C. crassiscolex* occurs in a molluscan host may here be significant. It is worthy of note that the only other dilepid cysticercoid described from a mollusc, viz. *Liga facilis* (Dilepidinae) in Macrochlamys (Spasskyi & Kasyanov, 1954) bears a marked resemblance to *C. crassiscolex*. Also the unusual larva of the dilepid Gryporhynchus is found in a reptile and a mammal. All of these hosts are less usual ones for cysticercoids.

for the early post-hexacanth stage of *C. crassiscolex*. Within the Dilepididae, subfamily Dipylidinae to which it belongs, a 'tailed' larva with a scolex has been described for *Dipylidium caninum* by Grassi & Rovelli (1889) and Venard (1938). The larva of *D. caninum* occurs in the haemocoel of a flea and no cyst is figured by either writer. Grassi & Rovelli show hexacanth hooks on the 'tail' of this larva and compare it with a cercaria. Venard has drawn an early stage with a long, blunt 'tail' and a scolex with suckers but no rostellum. He makes no comment on the significance of this stage. He figures also two further stages resembling those of our photographs for 3rd- and 4th-stage larvae but does not mention a cyst wall. In the former of these drawings he has drawn what looks like a bladder around the scolex, but he does not interpret it this way and regards what we think is the invaginated rim of the bladder as a posterior excretory pore, with our 'scolex cavity' as part of the excretory system opening into it. This reverses his orientation compared with ours, and leads him to regard the 'tail' as having been lost and the scolex to be an invaginated one. This latter point contradicts the accepted definition of a cysticercoid. We think that judging by the size and proportions of his figures, part of the 'tail' even here has become a bladder wrapped around the scolex. We draw attention to this figure as it has been chosen as an illustration by Hyman (1951). Whether or not a stage equivalent to our stage 1 exists in the closely related *Choanotaenia infundibulum* is not known, as Reid & Ackert (1937) did not describe one, though their final stage larva resembles ours closely.

Against the argument that there might be in *C. crassiscolex* a free larva resembling a plerocercoid at the post-hexacanth or pre-cystic stage there seemed to us to be two apparent pieces of evidence. First, there is the isolated instance described by Joyeaux (1922) of *Urocystis prolifer* larvae, from earthworms and myriapods, in which a multiple exogenous budding cyst forms numerous 'tailed' cysticercoids developing from the encysted hexacanth. Such cysticercoids could never have been free. The drawings given, however, do not convince us that the 'tails' are anything other than stolons arising from the cyst wall of the branched hexacanth. We also questioned our interpretion on examining the recent work of Kisielewska (1958) on the cysticercoids of *Choanotaenia crassiscolex* itself. In this paper, hexacanths and early stages of scolex formation are drawn—stages we were unable to find. It is not stated where in the snails they occurred, but they were presumably in the digestive gland since according to the text 'the entire morphogenesis of *C. crassiscolex* larvae in the intermediate host takes place inside the cuticular membrane'. The latter term and 'cuticular capsule' denote the cyst wall and the term 'cyst' is used to describe the bladder. The labelling in this work is incomplete and the methods of representing equivalent parts in the various drawings are not uniform. It would seem that the author regards the cyst wall as a larval cuticle but omits any explanation of when a second cuticle arises. From our sections of the mollusc it seems highly probable that the cyst wall and the muco-polysaccharide zone are host structures. In addition we query Kisielewska's drawing of the simultaneous development of scolex and bladder if this worm is in fact the one we describe, as there is no doubt at all about the early formation of a complete

scolex, together with a mobile 'tail' in our 1st-stage larva, and that the bladder arises later first from a part and then the whole of the 'tail' (minus any part that might have borne hexacanth hooks). Whether or not this differentiation takes place within the host cyst we do not know. We are not convinced that the 'cuticular capsule' around the hexacanths in Kisielewska's drawings is equivalent to our cyst wall of host tissue which she must have seen at the later stages. Consequently we do not consider that this work eliminates the possibility of a free plerocercoid stage. We have investigated the possibility that the parasite might have arrived at the site of encystment by way of the ciliated hepatic vestibule and the ducts of the digestive gland. If this had happened it would have been feasible to think that only a highly differentiated larva of this sort could navigate such a path.* But the evidence of the exact location of the parasite in the parts of the digestive gland immediately adjacent to the intestine, and never associated with the posterior digestive gland, points to the more likely possibility that the parasite reaches the site of encystment at the hexacanth stage by penetrating the intestinal wall. As we did not find any stage earlier than that shown in Text-fig. 1, the duration of any mobile highly differentiated post-hexacanth and precystic stage, if it occurs, must therefore be very short.

SUMMARY

1. Four stages in the development of *Choanotaenia crassiscolex* are described with the help of five text figures and one plate.

2. The functional anatomy of the cysticercoid is given with the help of diagrams.

3. The identification is based on comparisons with material at the British Museum of Natural History, South Kensington, London, and comparison is made with earlier records in Table 1.

4. The unusual cyclophyllidean scolex is discussed and tentative suggestions and queries concerning the general form of the early cysticercoid are put forward.

5. An attempt is made to determine more precisely than has hitherto been done for cysticercoids, the relationship of parasite and host.

The authors would like to thank Dr F. G. Rees, University College of Wales, Aberystwyth, for reading the manuscript and for making helpful criticism. Thanks are also due to Mr S. Prudhoe, British Museum of Natural History, London, for his valuable assistance with identification, and to Mr N. C. Davey, Senior Technician, Queen Elizabeth College, who carried out the photographic work.

* Rigby, J. E. (Ph.D. Thesis, 1960, Univ. of Reading).

LIST OF ABBREVIATIONS

a.l.	anterior lobe of sucker	*l.m.r.*	longitudinal muscles of rostellum
b_1	bladder	*l.m.s.*	superficial longitudinal muscles
b_2	blade of hook	*l.n.n.*	lateral nerve of rostellum
b.w.i.	inner wall of bladder	*m.p.*	mucous plug
c.	cuticle	*m.p.z.*	muco-polysaccharide zone
c.b.	cuticle of bladder	*m.m.*	meridional muscles
c.sc.	cuticle of scolex	*m.s.*	sucker muscles
c.sp.	cuticular spines	*n.*	neck
c.g.	cerebral ganglion	*o.m.s.*	superficial oblique muscles
c.m.r.	circular muscles of rostellum	*p.*	parenchyma
c.m.s.	superficial circular muscles	*p.a.n.*	primary anterior lateral longitudinal nerve
c.w.	cyst wall		
d.g.a.	anterior digestive gland	*r.*	rostellum
d.l.	delimiting layer of sucker	*r.m.*	radial muscles
d.m.	diagonal muscles	*r.c.*	rostellar capsule
e.m.	equatorial muscles	*ret.m.*	retractor muscle
g.	guard of hook	*r.n.*	rostellar nerve
g.c.	gland cells	*r.s.*	rostellar sac
h.a.	hook attachment	*s.*	sucker
h.a.c.	anterior circle of hooks	*s.a.n.*	secondary anterior longitudinal nerve
h.p.c.	posterior circle of hooks		
h.r.	rostellar hooks	*sc.*	scolex
i.	intestine	*sc.c.*	scolex cavity
l.a.	anterior lobe of sucker	*sc.sp.*	scolex sphincter
l.ex.v.	lateral excretory vessel	*s.m.*	superficial muscles
l.m.a.	attachment of longitudinal muscles	*t.*	tail
l.m.i.d.	inner deep longitudinal muscles	*ten.*	tendon
l.m.o.d.	outer deep longitudinal muscles	*t.m.*	transverse muscles

REFERENCES

Benham, W. B. (1900). The structure of the rostellum in two new species of tapeworm from Apteryx. *Quart. J. Micr. Sci.* **43**, 83–96.

Cohn, L. (1898). Untersuchungen über das centrale Nervusystem der Cestoden. *Zool. Jb.* Abt. *Anat.* **12**, 89–100.

Furhman O. (1928). Handbuch der Zoologie. *Kukenthal.* Band **2**, Teil 2.

Grassi, B. & Rovelli, G. (1889). Embryologische Forschungen an Cestoden. *Zbl. Bakt.* Band **5**, 370–7, 401–10.

Harper, W. F. (1930). On some British larval cestodes from land and freshwater invertebrate hosts. *Parasitol.* **22**, 202–13.

Hyman, L. H. (1951). *The Invertebrates.* Vol. II. McGraw-Hill Book Company, Inc.

Joyeaux, Ch. & Baer, J. G. (1936). *Faune de France*, **30**, *Cestodes*, Paris.

Joyeaux, Ch. (1922). Recherches sur l'*Urocystis prolifer*. *Bull. Soc. zool. Fr.* **47**, 52–8.

Kisielewska, K. (1958). The life cycle of *Choanotaenia crassiscolex* (Linstow 1890) (Dilepididae) and some data relating to the formation of its cysticercoids. *Bull. Acad. Polonaise Sci.* Cl. II, vol. VI, no. 2, 79–84.

Reid, W. M. & Ackert, J. E. (1937). The cysticercoid of *Choanotaenia infundibulum* (Bloch) and the housefly as its host. *Trans. Amer. Micr. Soc.* **56**, 99–104.

Rigby, J. E. & Rawson, D. (1958). A new type of early cysticercoid stage in a cyclophyllidean cestode. *Nature, Lond.* **182**, 121–2.

Schiller, E. L. (1959). Experimental studies on morphological variation in the cestode genus *Hymenolepis*. 1. Morphology and development of the cysticercoid of *H. nana* in *Tribolium confusum*. *Exp. Parasitol.* **8**, no. 2, 91–119.

Smyth, J. D. (1947). The physiology of tapeworms. *Biol. Rev.* **22**, 214–38.

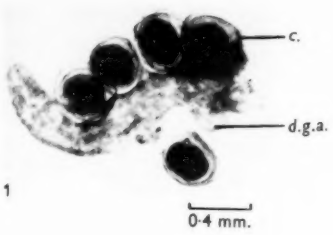
Spasskyi, A. A. & Kasyanov, I. S. (1954). Terrestrial molluscs as intermediate hosts of dilepidids in Kirghizia. *Trud. Helminth. Lab.* **7**, 185–7 (in Russian).

Venard, C. E. (1938). Morphology, bionomics, and taxonomy of *Dipylidium caninum*. *Ann. N.Y. Acad. Sci.* **37**, 273–328.

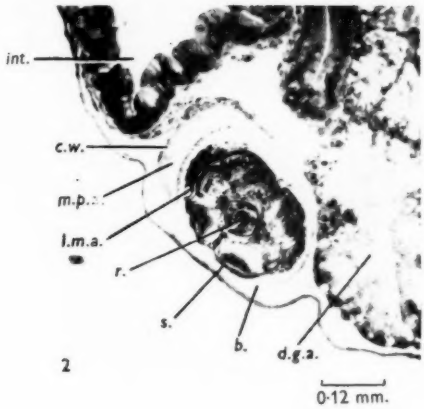
EXPLANATION OF PLATE 1

Plate 1 shows various stages and aspects of the cysticercoid larva of *Choanotaenia crassiscolex* in the digestive gland of *Oxychilus cellarius*.

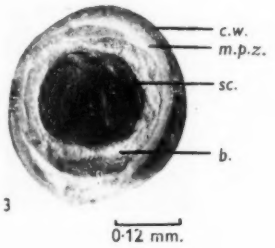
Fig. 1. Cysts in digestive gland.
Fig. 2. Section of gut and digestive gland showing cysticercoid in transverse section.
Fig. 3. 2nd-stage larva in cyst.
Fig. 4. 2nd stage larva showing tail inside ruptured cyst.
Fig. 5. 3rd-stage larva with tail stump—cyst removed.
Fig. 6. 3rd-stage larva. Tail almost completely withdrawn into bladder.
Fig. 7. 4th-stage larva—normal position in cyst.
Fig. 8. 4th-stage larva—cyst wall ruptured—larva straightened.



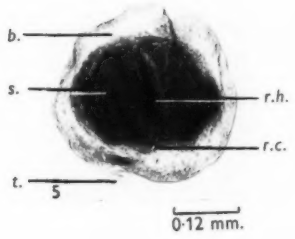
1



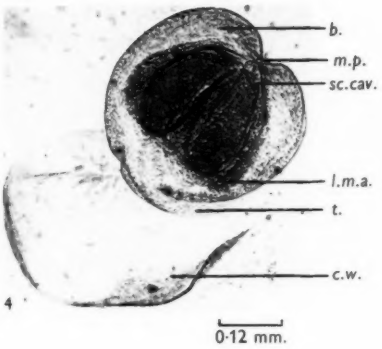
2



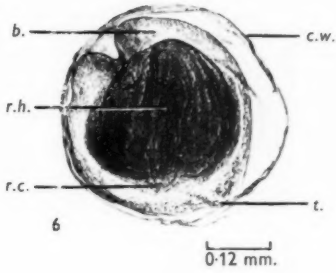
3



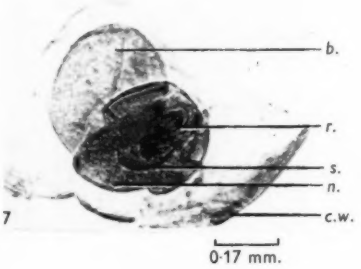
5



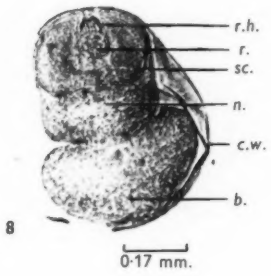
4



6



7



8

(*Facing p.* 468)

Parasitology (1960), **50**, 469–480
With 8 text-figures
Printed in Great Britain

Development of *Carmyerius exoporus* Maplestone (Trematoda: Gastrothylacidae) in a snail host

By J. A. DINNIK and N. N. DINNIK
East African Veterinary Research Organization, Muguga, Kenya

(*Received* 25 *January* 1960)

MATERIAL

Carmyerius exoporus Maplestone, 1923 has been described from the stomach of a sitatunga antelope, *Limnotragus spekii* (Sclater) taken in Nyasaland, Africa. This worm, which is easily distinguished from the other species of the genus *Carmyerius* found in Africa by the fact that the genital pore opens outside the ventral pouch, was first recorded in cattle by Solomon (1932), who found ten specimens of this species in material collected from the rumen of an ox at Thomson's Falls in Kenya. We found that *C. exoporus* is not uncommon in cattle and also occurs occasionally in sheep and goats in the Highlands, where its intermediate host is *Anisus natalensis* (Krauss, 1848). This small snail, which has so far been generally referred to the genus *Gyraulus*, is common on the vegetation found in shallow water in East, Central and South Africa (Mandahl-Barth, 1954).

The present observations were carried out to study the larval development of *Carmyerius exoporus* and, in particular, the succession of the redial generations in a species belonging to the family Gastrothylacidae Stiles & Goldberger, 1910. For all the following experiments, specimens of *C. exoporus* were collected initially from the rumen of a Zebu bull from the Highlands of Kenya. Laboratory-bred specimens of *Anisus natalensis* were exposed to miracidia which had hatched from eggs obtained from these worms and, when the snails began to emit cercariae, another animal, a 5-months' old calf, was given 928 encysted cercariae. The calf started passing *Carmyerius exoporus* eggs 100 days later and has since been used as a permanent source of eggs for the experimental infections of snails.

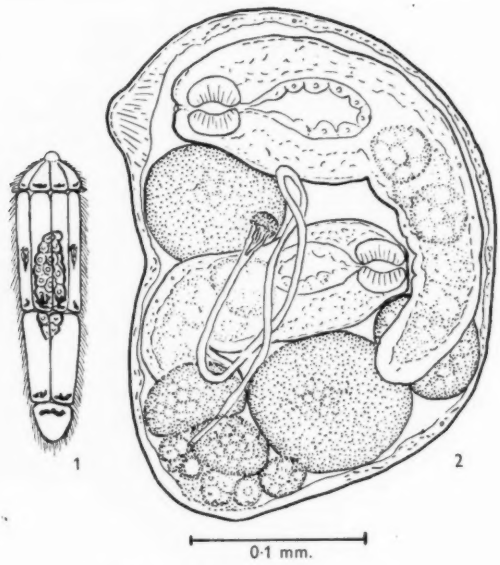
The snails used in the experiments were laboratory-reared specimens of *Anisus natalensis*, which were found to be very susceptible to infection at any age. In very young small specimens, however, the larval development of *Carmyerius exoporus* progressed more slowly and the death-rate was higher than it was when older snails were used. The snails were exposed either to a single miracidium or to three to five miracidia each and were dissected later, at appropriate intervals of time, to study the larval development in them. In addition, a number of snails was infected by placing them in a container with numerous swimming miracidia.

After observations on the development in the snails had been completed, the calf was sacrificed, 1 year and 9 months after it had been given 928 encysted cercariae, and 836 mature specimens of *C. exoporus* were recovered from its rumen.

DEVELOPMENT OF THE MIRACIDIUM AND SPOROCYST

The eggs of *C. exoporus* are 0·121–0·140 mm. long and 0·069–0·075 mm. wide. They are covered with a thin colourless shell provided with a delicately outlined operculum at one end and, as in most paramphistomes, each newly passed egg contains an embryo, composed of from one to several cells, situated in the centre amidst the yolk material of the egg. The miracidia develop in about 15 days and hatch over a period of several days after the first miracidium emerges.

The miracidium of *C. exoporus* measures about 0·165 mm. in length and 0·025 mm. in breadth at the shoulders and is of the usual paramphistome type, covered with twenty ciliated epithelial cells arranged in four transverse rows of,



Figs. 1–2. *Carmyerius exoporus*

Fig. 1. Miracidium, showing the group of germinal cells and the arrangement of the epithelial cells.

Fig. 2. Ten-day old sporocyst.

respectively, six, eight, four and two cells each (Fig. 1). Three glands are seen in the anterior end of the body in front of the nerve mass and a flame cell, directed postero-dorsally, is present on each side of the body cavity. The excretory ducts run posteriorly from the flame cells first, then turn back, form a loop and finally run posteriorly again to the excretory pores, which open at the junction of the 3rd and 4th rows of epithelial plates. A group of germinal cells is packed together and lies in a compact elongated mass in the body cavity. This group can be easily broken up into separate cells if a vitally stained miracidium is pressed under a cover-slip. It can then be seen that the group is composed of twenty-five to thirty-two germinal cells, which are somewhat irregular in shape. Each germinal cell

has a large nucleus surrounded by thin, deeply staining cytoplasm. A few small nuclei, resembling the nuclei of the body tissue and probably the nuclei of the syncytium, can be seen scattered among the germinal cells. Two bodies formed of granulated protoplasm, in which round cells with large nuclei are seen floating freely, extend from the posterior end along the dorsal and ventral sides of the body cavity. The floating cells resemble the germinal cells in structure but we could not ascertain their significance in the body of the miracidium or follow their development in the sporocyst.

After their penetration into a snail the miracidia, or the young sporocysts developed from them, were usually found to be localized in tissues surrounding the intestine or attached to the mantle of the snail. Rarely the sporocysts were seen deep in the mantle tissue and in the liver.

For the first 3 days the young sporocyst retained the shape of the miracidium from which it had originated. The germinal cells in its body began to grow into embryo balls, but we could not ascertain their number at this early stage. From the 6th to the 9th day the largest of these embryo balls in the sporocyst developed into the first redia, and 10–12 days after penetration of the miracidia into the snails the sporocysts were found to contain from one to four rediae each (Fig. 2). By that time the bodies of the sporocysts were broad and oval in shape, measuring 0·182–0·333 mm. in length and 0·104–0·195 mm. in width. The rediae seen in the sporocyst varied from very young specimens 0·14 mm. long to specimens 0·20–0·28 mm. long which were ready to leave the parent sporocyst.

It was not possible to see and count with any degree of certainty all the embryo balls in the body cavity of a sporocyst at this stage, since the body wall was no longer as transparent as it was in the earlier stages and large balls in the body cavity often concealed smaller balls behind them. Therefore, in order to count the embryos, twenty-five sporocysts were taken from several snails, before the first rediae which had developed in them could escape, and were dissected separately. In all the sporocysts, redial embryos and embryo balls were counted, using a microscope, and it was found that the number of embryos developed varied from six to eleven per sporocyst (Table 1).

Table 1. *The number of embryos developed in a sporocyst of* Carmyerius exoporus

Number of embryos per sporocyst	6	7	8	9	10	11
Number of sporocysts	4	5	7	4	3	2

We wished to determine whether all the embryo balls were capable of development into rediae after the first redia had ruptured the wall of the sporocyst and escaped from it, leaving the other embryos in the damaged sporocyst or scattered in the surrounding tissue of the snail. Twenty-five snails, each infected with a single miracidium, were dissected 15–17 days after exposure and the rediae recovered from each snail were counted. By that time it was expected that all the embryo balls would have developed into rediae, but that the first rediae would still be too young to have given birth to daughter rediae and thus, all the rediae found in a snail could only be those developed from a single sporocyst. It was

found (Table 2) that the number of rediae which developed in a snail infected with a single miracidium varied from three to nine.

Comparison of the numbers of germinal cells and embryo balls seen in the sporocyst and the number of rediae developed from them has shown that, from the twenty-five to thirty-two germinal cells present in the miracidium, only six to eleven developed into embryo balls in the sporocyst, and not all of these were able to complete their development into young rediae after the parent sporocyst was ruptured by the first redia. Some of the germinal balls which remained in the damaged sporocyst or which were scattered in the surrounding tissue of the snail perished and, out of six to eleven balls, only three to nine developed into rediae.

Table 2. *The number of rediae developed by a sporocyst of* Carmyerius exporus *in a snail host infected with a single miracidium*

Number of rediae per snail	3	4	5	6	7	8	9
Number of snails	4	5	3	3	5	3	2

The development of the sporocysts and the rediae in them was affected by the size of the infested snails, by the character of the snail tissue in which the miracidia had penetrated and by the degree of infestation of the snail. This development was delayed if the snail was young and too small or if the sporocyst was developing in the compact tissue of a snail. A single sporocyst in the snail produced all its rediae and then died in 10–13 days; on one occasion only the remains of a ruptured sporocyst were found on the 18th day in a snail exposed to one miracidium. When the snails were exposed to many miracidia the development of the sporocysts and rediae in them took 15-16 days and ruptured sporocysts were seen in the snails up to 23 days after their exposure to miracidia. When many sporocysts were cramped together in the compact tissue of a snail they did not develop normally and died a few days after the initial penetration of the miracidia into the snail. More than nine sporocysts were never seen in a snail, even in those which were exposed to numerous miracidia.

DEVELOPMENT OF THE REDIAE

The first free rediae of the first generation, i.e. the young rediae which had escaped from the parent sporocysts, were observed 9 days after exposure of the snails to a single miracidium each and somewhat later, on the 12th day, in the snails exposed to several miracidia each. The young rediae were 0·20–0·29 mm. long and contained germinal cells at the posterior end which had already given rise to a few embryo balls in the body cavity. The rediae grew in size and the embryo balls in them increased in number quickly so that in snails dissected 15–18 days after exposure rediae were found containing up to twelve embryo balls, of which the most advanced had developed into cercarial or redial embryos. By that time some rediae had grown up to 0·63 mm. long, but young rediae about 0·25 mm. long containing only germinal cells and balls were present in the snails.

The redia of *C. exoporus*, which belongs to the family Gastrothylacidae, is morphologically similar to the rediae of the family Paramphistomatidae and does

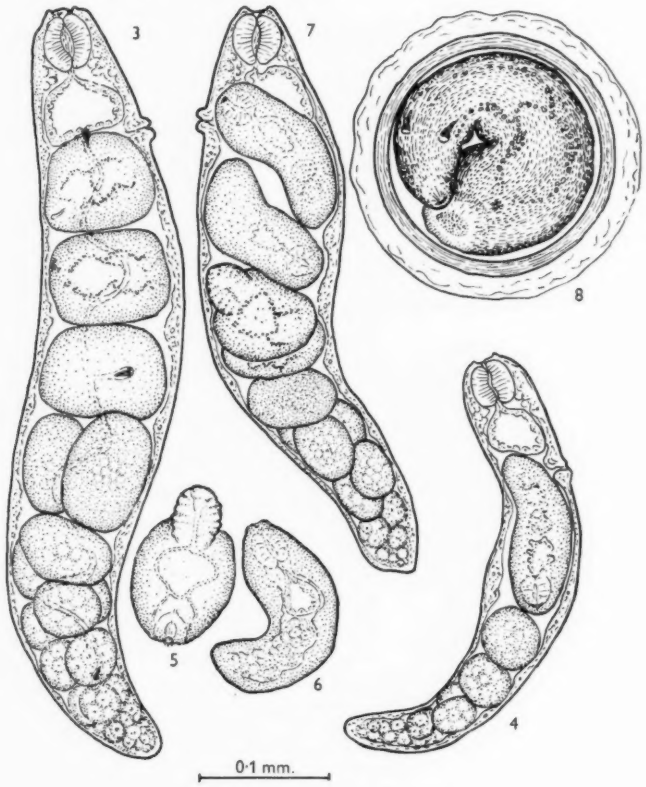
not possess a collar or any lateral appendages. The mature first generation rediae measure 0·35–0·65 mm. in length and 0·10–0·14 mm. in breadth. The muscular pharynx is 0·035–0·044 mm. long and the short sac-like gut extends to the level of or slightly posterior to the birth pore. There are three pairs of flame cells; the anterior pair is situated close to the posterior end of the gut, at the level of the birth pore, the second pair is in the middle of the body and the third pair is in the posterior part of the body. They form, with their ducts, the excretory system of the redia and the pores of this system open laterally midway along the body, a little anterior to the second pair of flame cells. Each mature redia contains a germinal mass and cells at the posterior end and embryo balls, tightly packed together in the middle part of the body cavity. The size of the balls gradually increases as they move towards the anterior part of the cavity, where developing daughter rediae and cercariae can be seen.

The cercariae leave the redia while they are still in the embryonic stage of development. A cercarial embryo can be recognized in the body cavity of the parent redia by its oral sucker and strings of small granules of the excretory system in its body and, at the more advanced stage, by its rudimentary tail. The daughter rediae also leave the parent redia at an early stage in their development. However, owing to the elongated shape of these redial embryos and the lack of conspicuous features in them at this stage of development, apart from the pharynx, it was not easy to recognize them while they were lying amongst large embryo balls in the body cavity of the parent redia. Only occasionally were young daughter rediae clearly seen in some of the parent rediae in whose body cavities they had delayed longer than usual, to the time when the pharynx, short gut and even small embryo balls had become clearly visible in their bodies. Rediae containing only cercarial embryos (Fig. 3), or only a redial embryo (Fig. 4), as well as rediae containing both cercarial and redial embryos were observed among mature rediae of the first generation recovered from the snail 15–20 days after its exposure to miracidia.

It was found, however, that it was not easy to differentiate cercarial and redial embryos in the body cavity of the parent redia. Quite often a redia which seemed to contain only cercarial embryos and embryo balls was found to contain a redial embryo also after it had been dissected and all its contents examined.

A special series of examinations was, therefore, carried out in which many infested snails were dissected 15–19 days after their exposure to miracidia. At this time the first embryo balls in the rediae developing in the snails had begun to form daughter rediae or cercariae but none had left the parent rediae. In a few snails, dissected on the 18th and 19th days of the infection, daughter rediae and developing cercariae, which had already left the parent rediae and continued their development free in the snail's tissue (Figs. 5, 6), were observed but such snails were excluded from this series. Only mature rediae recovered from those snails in which neither free daughter rediae nor cercariae were seen were used. Because of the uncertainty in identifying redial embryos, while they are in the body of the intact parent redia, all the mature rediae recovered from the snails were dissected on glass slides and examined separately under a microscope. More

than a hundred mature rediae were dissected and examined and it was found that in about 95% of them the first three to six embryo balls were developing into cercariae and only in 5% was the first ball seen to be developing into a redia while the younger balls were cercarial embryos.



Figs. 3–8. *Carmyerius exoporus*

Fig. 3. First-generation redia containing cercarial embryos, from a snail 15 days after its exposure to a miracidium.

Fig. 4. First-generation redia containing a daughter redia, from a snail 17 days after its exposure to a miracidium.

Fig. 5. Newly born cercaria.

Fig. 6. Newly born redia of the second generation.

Fig. 7. First-generation redia containing daughter rediae and cercarial embryos from a snail 25 days after its exposure to a miracidium.

Fig. 8. Encysted cercaria.

For example, in a snail dissected 17 days after exposure ten mature rediae were found but only in one of these was a redial embryo, which was followed by seven embryo balls, seen. In five other rediae from the same snail the first embryos were all cercarial, from one to three per redia, and four rediae still contained

unidentifiable embryo balls in the anterior part of the body cavity. Three other snails of the same series were dissected 19 days after exposure and thirty-six rediae recovered from them were dissected. Only in one was the first embryo redial; in all the other rediae the first embryos were cercarial or still unidentifiable balls. No redia was seen at this stage of development which contained more than one daughter redia.

Daily dissections of snails from the 15th day onwards showed that the first daughter rediae and cercariae escaped from the 18th to the 20th day, leaving an empty space in the anterior part of the body cavity of the parent redia. In a short time this space became filled with large new embryo balls which developed into new redial and cercarial embryos. These became recognizable 22–25 days after exposure of the snails to miracidia. By dissecting first generation rediae at that time we found specimens containing one or two redial embryos or mobile daughter rediae, followed by one to seven clearly recognizable cercarial embryos and embryo balls of various sizes in the body cavity (Fig. 7). The following are two examples of these dissections.

A snail exposed to several miracidia was dissected 22 days later. Twenty-seven rediae were recovered, of which fifteen were mature and four were immature rediae of the first generation and eight were new-born rediae of the second generation. Many young cercariae developing free in the snail tissue were also recovered. All fifteen mature rediae were dissected and their contents were as follows: one contained one redial embryo and eight embryo balls; one contained one daughter redia, one redial embryo and four embryo balls, and one contained daughter redia, two cercarial embryos and five embryo balls; in six rediae only cercarial embryos, from two to five in each, together with embryo balls, were found, while the other six rediae contained only embryo balls in the body cavity.

Another snail from the same series was dissected on the 25th day and eleven mature rediae, one of which was dead, five immature rediae of the first generation, four small young rediae of the second generation and many free, developing cercariae were found. Ten mature rediae were dissected and their contents were as follows:

(1) Three cercarial embryos and six embryo balls;
(2) One daughter redia, seven cercarial embryos and four embryo balls;
(3) Ten embryo balls;
(4) Five cercarial embryos and six embryo balls;
(5) Two cercarial embryos and eight embryo balls;
(6) One daughter redia, three cercarial embryos and five embryo balls;
(7) Five cercarial embryos and no embryo balls, but germinal cells were present at the end of the body cavity;
(8) Two daughter rediae, two cercarial embryos and eight embryo balls;
(9) Seven embryo balls;
(10) Four cercarial embryos and eight embryo balls.

All the rediae which were examined had many germinal cells at the end of the body cavity from which new embryo balls developed.

Further dissections of the rediae recovered from snails from the 25th day onwards have shown that most of the stream of embryo balls, which are formed at

the posterior end and pushed to the anterior part of the body cavity, develop into cercarial embryos and only a small number of these balls develop into redial embryos. No alternation in the development of redial and cercarial embryos was noted in the rediae of *C. exoporus*. Redial embryos were observed to develop alongside the more numerous cercarial embryos throughout the life of the 1st-generation rediae. It looked as though groups, each consisting of several balls, developed one after another throughout the life of the redia from germinal cells situated at the posterior end of the body cavity. In the first of these groups most of the embryo balls developed into cercarial embryos and a few balls, possibly not more than one, developed into redial embryos. When all, or most of the embryo balls of the first such group had developed and left the parent redia as young cercariae and rediae, a second group of embryo balls moved to the anterior part of the body cavity to develop in turn into numerous new cercariae and a few daughter rediae.

Redial embryos and daughter rediae were seen even in old parent rediae at the end of their life, when their body walls had become almost opaque and their contents were not clearly visible. Such old rediae of the first generation, containing small numbers of balls and embryos, were first seen 40 days after exposure of the snail to miracidia. In the snails dissected on the 50th to the 55th day, old rediae as well as dead rediae of the first generation were found frequently.

As has already been stated, the 2nd-generation rediae began to leave their parent rediae, and appeared free in the snail tissues, 18 days after the exposure of these snails to miracidia. The first of these rediae reached maturity and began to produce daughter rediae of the 3rd generation and cercariae on the 8th–10th day of their free life in the snail's tissue. The rediae of the 2nd and successive generations contained smaller numbers of embryos and were never seen to grow to the size of the large rediae of the first generation.

As a result of the combined production of the 1st and 2nd generation of parent rediae the number of rediae found in a snail increased progressively the longer the infestation lasted. The snails dissected between 15 and 17 days after exposure to a single miracidium each were found to harbour from three to nine rediae of the 1st generation, while the snails dissected much later, between 51 and 68 days after their exposure to a single miracidium each, were found to contain the following numbers of rediae, respectively.

In one snail dissected on the 51st day—32 rediae; in four snails dissected on the 54th day—29, 29, 41 and 32 rediae; in two snails dissected on the 55th day—34 and 26 rediae; in four snails dissected on the 59th day—19, 27, 24 and 24 rediae; in three snails dissected on the 63rd day—27, 31 and 20 rediae; and in two snails dissected on the 68th day—30 and 35 rediae.

When snails were exposed to several miracidia each, the number of rediae developed per snail was greater than it was in those infected with a single miracidium but none of them ever contained more than seventy rediae each. This is already a heavy burden for such a small snail as *Anisus natalensis*.

In snails dissected after the 70th day of the infestation no rediae of the 1st generation were present as they had all died by then. All rediae recovered

belonged to the 2nd or later generations and ranged from mature rediae with developing redial or cercarial embryos to new-born rediae with a few small embryo balls in the body cavity. At this time, quite late in the larval development in a snail host, it was impossible to distinguish the different generations of rediae with any certainty. The mature rediae recovered from a snail dissected 75 days after exposure were 0·28–0·38 mm. long and each contained from four to six large embryo balls plus a few small ones in their body cavities. In most rediae the embryo seen in the anterior part of the body cavity was cercarial and only in a few rediae was a redial embryo seen.

Even lightly infested *A. natalensis* could not survive long and the mortality amongst them increased to a very high rate after the 65th day of the infestation. Out of many hundreds of infested snails eleven survived to the 95th day, but only four were alive on the 120th day of their infestation, when they were dissected. These latter four snails were found to contain respectively the following:

(1) Eleven mature rediae with cercarial embryos and embryo balls, four young rediae, two new-born rediae and nineteen free developing cercariae.

(2) Eight old and dying rediae, thirteen rediae with cercarial embryos and embryo balls, three young rediae and twenty-three free-developing cercariae.

(3) One dead redia, one redia with a redial embryo and embryo balls, six rediae with cercarial embryos and embryo balls and one young redia, and eighteen free-developing cercariae.

(4) Two old dying rediae, four rediae with cercarial embryos, eight young rediae, 1 new-born redia and twenty-two free-developing cercariae.

The mature rediae, recovered from the snails dissected 120 days after exposure to miracidia, were 0·23–0·28 mm. long and contained from three to six embryos, one of which was a recognizable cercarial or redial embryo, and a small group of germinal cells at the posterior end of the body cavity.

THE CERCARIAE

In *Carmyerius exoporus* the cercariae leave their parent redia when they are still immature and for quite a long time they continue their development to maturity in the liver of the snail host. In this experimental series immature cercariae which had emerged from the parent rediae of the 1st generation were first observed 18 days after exposure of the snails to miracidia.

Immature cercariae leave the parent redia through its birth pore. The bodies of such newly born cercariae measured from 0·08 to 0·13 mm. in length, and their tails varied from rudimentary appendages to structures about half of the body length. At this early stage of free development of the cercaria in the snail tissue the oral sucker was clearly seen, with the mouth encircled by numerous tiny papillae, but the posterior sucker was still unrecognizable. In the body, which at this early stage of the free development contained no granules or rods of the cystogeneous cells, the main ducts of the excretory system were clearly seen and were of the same pattern as in the cercariae of Paramphistomatidae. The caeca were short and took the form of two broad diverticula at the end of the long oesophagus.

A pair of pigmented spots, which were not seen in the cercariae while they were still in the body cavity of the parent rediae, became prominent shortly after the immature cercaria emerged. As the cercaria grows in size its posterior sucker becomes visible and the cystogenous cells begin to produce granules and rods in the external layer of the body. The pigment starts to grow out from the pair of pigmented spots in irregular dendritic branches which spread over the surface of the cercaria's body and gradually cover it all, except the tail, with dark brown pigment. This pigment, together with the granules and rods of the cystogeneous glands, obscures the internal structure of the mature cercaria. In this mature cercaria the two eye-spots become prominent and are seen to be distinct conical structures, each with a refractile lens. During its development to maturity the body of the cercaria grows from 0·80 mm. to 0·25–0·30 mm. in length and its tail becomes 0·50–0·70 mm. long.

Mature cercaria began to emerge from the infected snails 31–33 days after their exposure to miracidia. Thus it appeared that, at 26° C., the development of the cercaria up to the time of its escape from the parent redia took about 9 days while its further development from birth to maturity in the snail tissue required about 14 days.

In our experiments infected specimens of *Anisus natalensis* emitted small numbers, not more than twelve, cercariae daily. Usually they only shed a few; a count of the cercariae emitted by the snails between the 43rd and 60th day of their infestation showed that only three to eight cercariae per snail were shed daily.

After the cercaria has left the snail it swims for some time, usually for about ½ hr., in the water. When swimming, by means of vigorous lashings of its long tail, the body of the cercaria becomes ball-shaped, with the two bulging eyes directed forwards and it moves towards the light. The cercariae attached themselves to the wall of the glass container or to the leaves of plants near the water surface.

The cyst of *Carmyerius exoporus* is round if viewed from above and dome-shaped in lateral view (Fig. 8). The diameter of the cyst itself is 0·219–0·262 mm. and its slightly widened base, by which it is supported and attached to the substratum, measures from 0·268 to 0·326 mm. in diameter. Its wall is colourless and transparent but the metacercaria inside it retains the heavy pigmentation of the cercaria and, therefore, the cyst as the whole looks black or dark brown in colour. The metacercaria moves occasionally within the cyst and is usually folded in such a way that the lateral surface is partly visible.

DISCUSSION

Judging from these observations on the development of *C. exoporus* as well as from those on the development of *Gastrothylax crumenifer* (Creplin) and *Carmyerius dollfusi* Golvan, Chabaud et Gretillat, which were carried out by Tandon (1957) and by Gretillat (1959), respectively, it appears that the larval development of these species, belonging to the family Gastrothylacidae, is similar to that of species belonging to the familyParamphistomatidae.

The miracidia of *Carmyerius exoporus* and *C. dollfusi* are covered with twenty

ciliated epithelial cells arranged in four transverse rows of six, eight, four and two cells, respectively, as is the case in those species of Paramphistomatidae whose miracidia are known. There is not much difference in the internal structure of the miracidia or in their germinal material, which seems, in all species of both families, to be composed of many germinal cells packed together in a compact morula-like body. In all the species, in both families, whose development is known the miracidium gives rise to a single sporocyst in which the germinal cells develop into rediae. The size of the sporocyst and the number of rediae developing in it, however, varies in the different species. In all species of both families the rediae possess neither a collar nor lateral appendages and are so alike morphologically that they are indistinguishable from each other. The differences which exist between the sporocysts and rediae of the various species in the families Paramphistomatidae and Gastrothylacidae are no more than specific differences or specific adaptations to development in particular snail hosts.

Gretillat (1959) has stated that in *Carmyerius dollfusi* the daughter rediae leave their parent rediae at a very early stage in their development, when they are still in an embryonic stage, and that it is impossible to distinguish their inner structure. The same phenomenon has been noted in the daughter of rediae of *C. exoporus*, while in *Gastrophylax crumenifer*, and likewise in *Paramphistomum microbothrium* and *P. sukari*, the daughter rediae leave the parent redia at a more advanced stage of their development when the pharynx, the gut and even embryo balls in the body cavity can be clearly seen.

There is a difference in redial productivity among the species and we think that the redial and consequently the cercarial productivity of the rediae is related to the size of the snail species. In the comparatively large snail species such as *Bulinus tropicus* and *Biomphalaria pfeifferi*, which are the snail hosts of *Paramphistomum microbothrium* and *P. sukari*, respectively, the rediae of the first generation start by producing many daughter rediae and then begin to give birth to numerous cercariae (Dinnik & Dinnik, 1954, 1957). As a result the snails soon become burdened with hundreds of developing rediae and cercariae but, being large, they can withstand this burden successfully.

Small snail species like *Anisus natalensis*, whose flat spiral shell is about 5 mm. in diameter and 1 mm. in height, cannot withstand a burden of hundreds of rediae and cercariae developing and feeding on its body. As an adaptation to the limited space and supply of nourishment in its snail host the rediae of *Carmyerius exoporus* give birth to one daughter rediae at the beginning of their productivity and a few more later, after the first bunch of cercariae have emerged from the rediae. Similar adaptations to the size of their snail hosts can be noted in the larval development of *Ceylonocotyle streptocoelium* (Fischoeder), *Paramphistomum ichikawai* Fukui, studied by Durie (1953), and *Gigantocotyle explanatum* (Creplin) whose life history has been described by Kurwar Singh (1958). The snail hosts of all these species are *Planorbinae* of small size and in all these species of the family Paramphistomatidae, as in *Carmyerius exoporus*, which belongs to the family Gastrothylacidae, only a few daughter rediae were seen to develop in the rediae of the first generation.

In both these families cercariae leave the parent redia when they are still immature and complete their development to maturity in the liver of the snail. Although this development seems to proceed similarly in all the species it appears that the differentiation leading to the development of the distinct morphological features of the adult worms which distinguish the two families one from the other begins during the course of cercarial development. Much could be learned, therefore, from a comparative study of the morphology of the cercariae of Paramphistomatidae and Gastrothylacidae and their development into the adult worms which might help to clarify the relationship of these two families, whose species differ so distinctly in the adult stages but are so alike in their larval stages.

SUMMARY

The development of *Carmyerius exoporus* Maplestone in the snail *Anisus natalensis* Krauss is described with the emphasis laid on the redial and cercarial productivity of the rediae of the first and subsequent generations.

The miracidium gives rise to one sporocyst. From 25 to 32 germinal cells present in the miracidium, only six to eleven develop into embryo balls in the sporocyst and, of these, only three to nine develop into rediae of the first generation.

The redia of the first generation gives birth to daughter rediae and cercariae. No alternation in the development of redial and cercarial embryos was noted in the rediae of *C. exoporus* and a small number of redial embryos was observed to develop alongside the more numerous cercarial embryos throughout the life of the first generation rediae.

The rediae of the second and subsequent generations also give birth to daughter rediae and cercariae.

The cercariae leave their parent redia when they are still immature and continue the development in the snail tissue.

REFERENCES

DINNIK, J. A. & DINNIK, N. N. (1954). The life cycle of *Paramphistomum microbothrium Fischoeder*, 1901 (Trematoda: Paramphistomidae). *Parasitology*, **44**, 285–99.

DINNIK, J. A. & DINNIK, N. N. (1957). Development of *Paramphistomum sukari* Dinnik, 1954 (Trematoda: Paramphistomidae) in a snail host. *Parasitology*, **47**, 209–16.

DURIE, P. H. (1953). The paramphistomes (Trematoda) of Australian ruminants. II. The life history of *Ceylonocotyle streptocoelium* (Fischoeder) Nasmark and of *Paramphistomum ichikawai* Fukui. *Aust. J. Zool.* **1**, 193–222.

GRETILLAT, S. (1959). Recherches sur le cycle evolutif de *Carmyerius dollfusi* Golvan, Chabaud et Gretillat, 1957 (Trematoda: Gastrothylacidae) à Madagascar. *C.R. Acad. Sci., Paris*, **248**, 1873–5.

MANDAHL-BARTH, G. (1954). The freshwater molluscs of Uganda and adjacent territories. *Ann. Mus. Congo belge*, 8vo **32**, 7–206.

MAPLESTONE, P. A. (1923). A revision of the Amphistomata of mammals. *Ann. Trop. Med. Parasit.* **17**, 113–212.

SINGH, K. S. (1958). A redescription and life-history of *Gigantocotyle explanatum* (Creplin, 1847) Nasmark, 1937 (Trematoda: Paramphistomidae) from India. *J. Parasit.* **44**, 210–24.

SOLOMON, S. G. (1932). On a collection of parasitic worms from East Africa. *J. Helminth.* **10**, 209–30.

TANDON, R. S. (1957). Life history of *Gastrothylax crumenifer* (Creplin, 1847). *Z. wiss. Zool.* **160**, 39–71.

Parasitology (1960), **50**, 481–496
With 16 text-figures
Printed in Great Britain

A comparative account of the tracheal system of larvae of the horse bot-fly, *Gasterophilus intestinalis* (De Geer), and of some other dipterous larvae

By R. J. TATCHELL
The Molteno Institute, University of Cambridge

(*Received* 3 *February* 1960)

The larvae of *Gasterophilus* live in an environment where the oxygen tension approaches zero and where the concentration of carbon dioxide is 70–80% (Tappeiner, 1883) and have been shown to tolerate anaerobic conditions, *in vitro*, for long periods (von Kemnitz, 1916; Dinulescu, 1932) and to depend on the maintenance of a high internal concentration of carbon dioxide (Levenbook, 1951). The larvae also possess an intracellular haemoglobin and it was shown by Keilin & Wang (1946) that, because of its very high affinity for oxygen, the haemoglobin would release any bound oxygen only at very low oxygen tensions. The haemoglobin is present in most larval tissues but is concentrated in certain modified fat-body cells of the so-called 'tracheal organ' or 'organe rouge'. These modified fat-body cells of the tracheal organ with their dense weft of ramifying intracellular tracheoles have been described, many times, in great detail (Enderlein, 1899; Prenant, 1899, 1900; Portier, 1911; Dinulescu, 1932; Keilin, 1944). The presence of this tracheal organ indicates that there has been considerable modification of the tracheal system in connexion with the storage of oxygen. So far as the tracheal system is concerned Enderlein (1899), Prenant (1900) and Portier (1911) describe only the large tracheae associated with the tracheal organ. The tracheal systems of other oestrid larvae have been only poorly described. Thus, Enderlein (1899) describes the tracheal organ and its cells of *Cobboldia* and *Gyrostigma* and Carpenter & Pollard (1918) give a very incomplete account of the tracheal system of *Hypoderma bovis*.

The tracheal systems of many dipterous larvae have been studied in great detail, but the account by Whitten (1955) is the only one that is complete and truly comparative. She showed the remarkable stability of the basic larval system regardless of the other anatomical variations of larval Diptera. The present investigation was undertaken with the object of finding the homologies of the tracheae of the tracheal organ and seeing what changes had occurred in the basic pattern.

MATERIALS AND METHODS

Larvae of *Gasterophilus intestinalis* were obtained from the stomachs of freshly slaughtered horses. Eggs, for the study of first instar larvae, were collected from the hides. Other larvae studied were those of *Drosophila* sp. and *Scatopse notata* from a local source and since maintained as a laboratory culture; *Eristalis tenax*

from a local source; *Calliphora erythrocephala* and *Aëdes aegypti* from long established laboratory cultures.

For the investigation of the tracheae of the larger forms, that is larvae of *Gasterophilus*, *Calliphora* and *Eristalis*, the cobalt sulphide injection technique devised by Wigglesworth (1950) was used. In the case of *Gasterophilus* it was necessary to open the lips of the spiracular pouch by ligation before proceeding with the technique. It was found more convenient to precipitate the CoS and fix the larvae at the same time by immersing them in Carnoy's fixative which had been saturated with H_2S by the addition of a drop of ammonium sulphide (R. Christopher's modification). The early instars of the large forms and all stages of the smaller ones were studied after compression of the living larvae between a slide and cover-slip in the gum-arabic medium of Faure (quoted in Langeron, 1949).

The descriptive terms used in the account are those of Whitten (1955) and her definitions have been retained exactly.

RESULTS

The tracheal system of larvae of Gasterophilus

The larval head is non-capsular, and, as with all other cyclorrhaphous larvae, the head segments have been invaginated into the mouth. This change is accomplished without any basic change in the tracheal system although certain detailed relationships have altered.

The first instar larva is metapneustic, as are the majority of cyclorrhaphous larvae at that stage. The postabdominal spiracles are borne at the end of two small projections of the last abdominal segment. The openings appear double and are supported by a number of Y-shaped stigmatic sclerites. From the spiracular openings long felt-chambers lead forward into the main body of the larva before joining the main dorsal longitudinal tracheal trunks. The remainder of the tracheal system (Fig. 1) shows a well-marked metamerism and can be readily homologized with Whitten's basic system. There are, however, minor differences in the tracheae of the anterior segments which are repeated in the later instars and will be mentioned in the description of the third instar, as will those differences in the arrangement of the posterior tracheae that will be incorporated in the tracheal organ.

The second instar larva is also metapneustic and although this is unusual, Keilin (1944) records that a similar condition with respect to the first two instars has been noted in *Thrixion halidayanum* Rond. described by Pantel (1898) and *Panzeria rudis* Fall. described by Nielsen (1909). The postabdominal spiracles are completely enclosed within a terminal invagination of the last abdominal segment, which forms, by two transverse folds of the larval integument, dorsal and ventral lips. These lips to the spiracular pouch are in communication with the haemolymph and by changes in their turgor pressure they may open to expose the spiracular plate or close and form a completely gas-tight seal. The spiracular plate may also, under muscular control, be partially extruded when the lips are fully retracted, so bringing it into direct contact with the medium. On each side of the spiracular

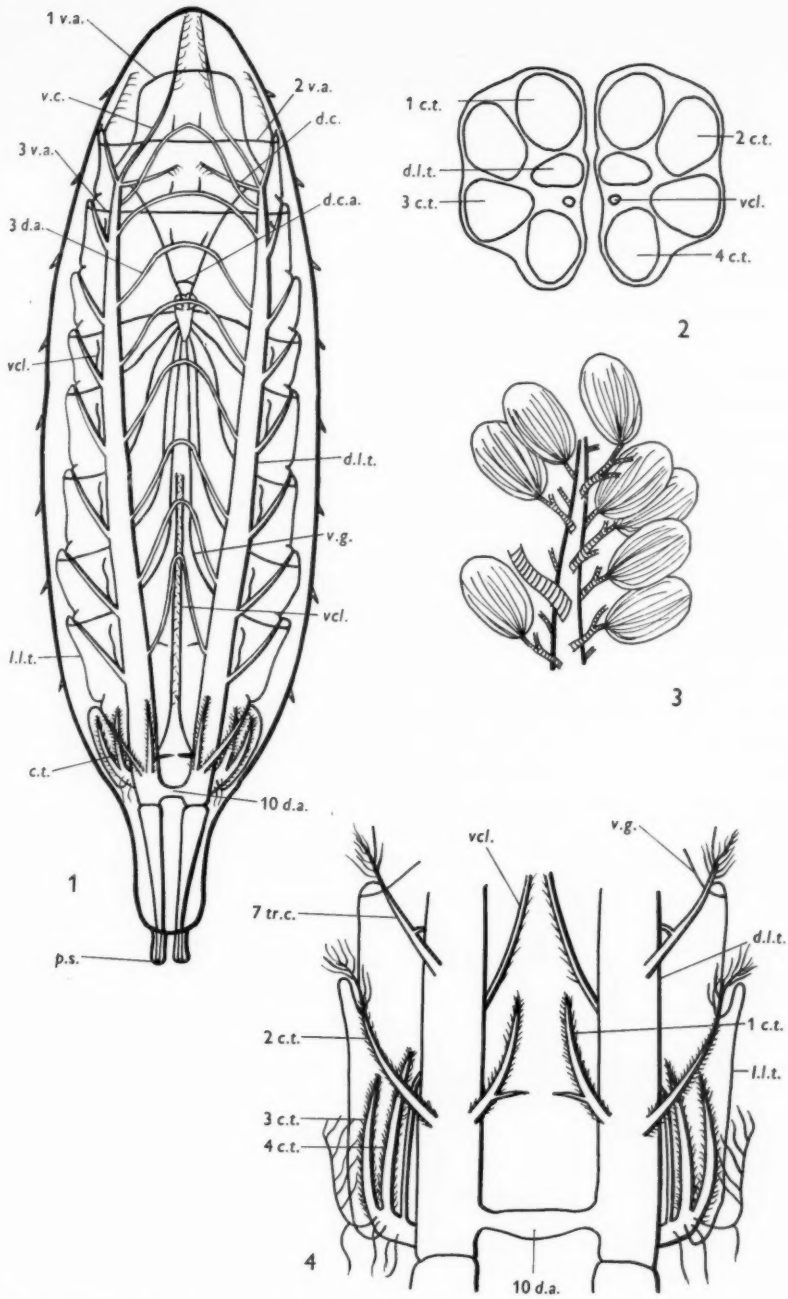


Fig. 1. The tracheal system of the first instar larva of *Gasterophilus*. Dorsal view.

Fig. 2. A plan of the tracheae given off from the inner face of the common spiracular chamber of *Gasterophilus*.

Fig. 3. A portion of the tracheal organ showing the relations between the haemoglobin containing cells and the intracellular tracheae originating from the conical tracheae.

Fig. 4. The posterior tracheae of the first instar larva of *Gasterophilus* showing the distribution of the tracheae of the developing tracheal organ. Dorsal view.

plate a small sensory papilla is to be found. A very exact description of the postabdominal spiracles of the third instar has been given by Portier (1911) and those of the second instar differ little except that there are only two pairs of curved slit-like openings into the felt-lined common spiracular chamber. The remainder of the tracheal system is identical in plan with that of the third instar.

The third instar larva is morphologically amphipneustic but functionally metapneustic. The anterior spiracles, which were well described by Keilin (1944), are embedded in a deep groove, or pit, of the larval cuticle and morphologically are fully developed. The experimental evidence, however, shows that until extruded at pupation they are not in free communication with the exterior. Levenbook (1951) showed that, when the posterior spiracles were ligated and the larvae placed in a vacuum, they were inflated by the contained air but there was no air loss. In fact, even the normal loss of CO_2, which occurred with closed abdominal spiracles and was assumed to take place across the cuticle, stopped. This was explained by the possibility of physical changes in the stretched cuticle resulting in a loss of permeability and the occlusion by compression of the long felt-chamber of the anterior thoracic spiracles by the excessive internal pressure. In the present investigation it was found that, using Wigglesworth's injection technique, it was impossible to introduce the dissolved cobalt naphthenate into the tracheal system when the posterior spiracles were not artificially opened. On occasion the cobalt naphthenate penetrated the cuticular groove as far as the anterior spiracle but no further. Throughout the third instar it becomes progressively easier to extrude the anterior spiracles to the position they occupy as the pupal 'horns'. Even with the spiracles extruded and with a series of graded pressures it still proved impossible to introduce any cobalt naphthenate into the tracheal system, except by cutting off the tip of the spiracular 'horn' and exposing the lumen of the felt-chamber. It seems, therefore, that the spiracle is effectively sealed off from the exterior throughout larval life and becomes functional only after extrusion, and perhaps owing to the associated drying, at the time of pupation.

The postabdominal spiracular plate consists of three pairs of curved slits with the external scar of the second instar spiracular plate located centrally. The inner surface of the plate is lined with a layer of felt which forms the posterior wall of the common spiracular chamber. Six pairs of unequal tracheae (Fig. 2) are given off from the anterior face of the common spiracular chamber. Located peripherally are the four pairs of large openings (1–4 *c.t.*) that lead into the tracheae of the tracheal organ. Two pairs of smaller openings (*d.l.t.*, *vcl.*) are situated centrally, of which the larger (*d.l.t.*) lead into the dorsal longitudinal trunks and the smaller (*vcl.*) into the posterior visceral tracheae.

The eight main tracheae of the tracheal organ are exceptionally wide at their bases, but as they run forward through the tracheal organ to the level of the penultimate abdominal segment they progressively taper and thus acquire a marked conical shape (Keilin, 1944). From all over their surface tracheoles are given off which branch and ramify to terminate eventually as intracellular tufts within the large haemoglobin-containing cells of the tracheal organ (Fig. 3). The

ventral and ventrolateral (4, 3 *c.t.*) pairs of large conical tracheae also give off large branches from near their bases which supply the musculature of the body wall in their locality. Similar tufts of smaller tracheae are given off from the tips of the dorsolateral and the dorsal (2, 1 *c.t.*) pairs of conical tracheae.

The remainder of the tracheal system is metamerically arranged and can be analysed into the following components (Fig. 5).

Dorsal longitudinal trunks (*d.l.t.*). These are two large tracheae running forward from the common spiracular chamber to the anterior thoracic spiracles. There is a regular diminution in diameter from behind forwards but without any of the great variations found in some other larvae (e.g. *Eristalis tenax, Aëdes aegypti*).

Lateral longitudinal trunks. A pair of these are present running the whole length of the larva. In the third instar, because of the confusion caused by the vast number of small tracheae supplying the body wall in this region, they cannot be traced further back than an apparent termination towards the tip of the dorsolateral conical trachea (2 *c.t.*) of each side. By analogy with the situation in the second instar, however, it is likely that a further branch connects the dorsolateral to the branch of the ventral conical trachea (4 *c.t.*) which supplies the body wall. Anteriorly they terminate, after giving off a large trachea to the dorsolateral musculature, by uniting with the extremity of the dorsal longitudinal trunk.

Transverse connectives (*tr.c.*). Seven pairs of these link the dorsal and lateral longitudinal trunks of each side.

Visceral tracheae (*vcl.*). With the exception of the second, each transverse connective gives off a visceral branch to supply the internal organs; their distribution is as follows.

Visceral trachea of first transverse connective to: posterior oesophagus and the anterior face of the proventriculus.

Visceral trachea of the third transverse connective to: salivary gland and fat-body.

Visceral trachea of the fourth transverse connective to: fat-body, salivary gland and proventriculus.

Visceral trachea of the fifth transverse connective: after forming the 'tracheal body' (Fig. 5 *t.b.*) (Keilin, 1944) it reforms and continues to the fat-body, salivary gland and the region of the mid-gut immediately posterior to the proventriculus.

Visceral trachea of the sixth transverse connective to: mid-gut anterior to the main coils.

Visceral trachea of the seventh transverse connective to: main coils of the mid-gut.

Whitten (1955) found, in *Aphiochaeta* and *Calliphora*, that the visceral trachea given off from the lateral longitudinal trunk just before its posterior anastomosis with the dorsal longitudinal trunk was very much larger than the others. In *Gasterophilus* a similar situation exists although this posterior visceral trachea arises directly from the common spiracular chamber rather than from the lateral longitudinal trunk and then supplies the rectum and the main intestinal coils.

Dorsal anastomoses (*d.a.*). These link dorsally the dorsal longitudinal trunks in each segment. The first two arise between the anterior spiracles and the first

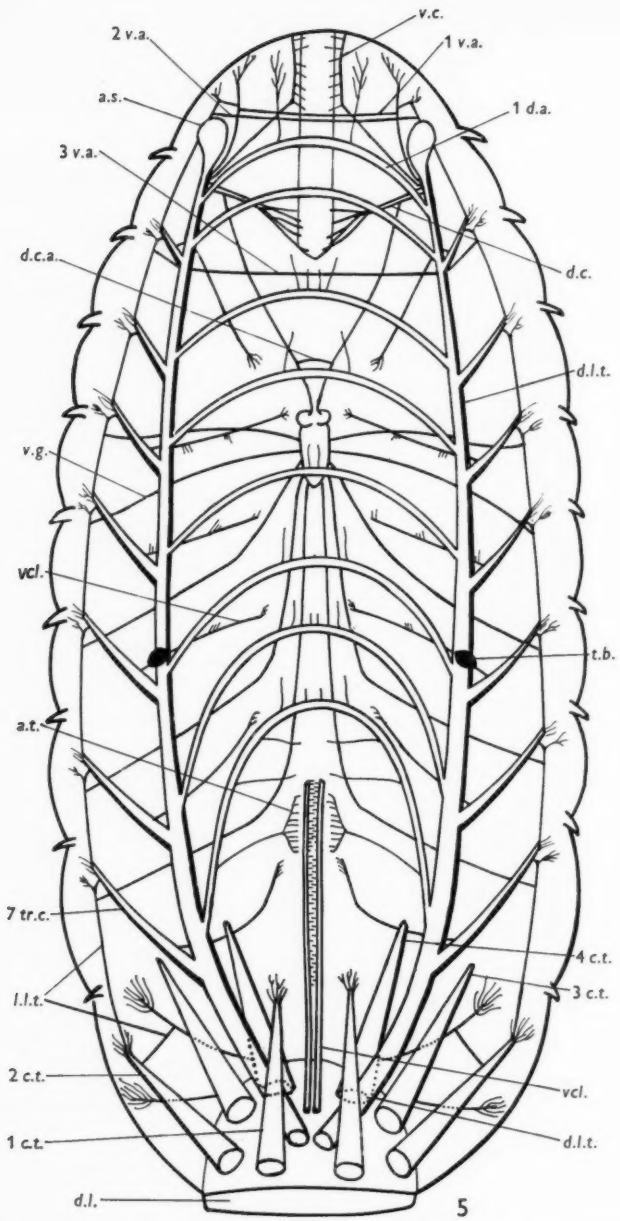


Fig. 5. The tracheal system of the third instar larva of *Gasterophilus*. Dorsal view.

transverse connective; the third to the eighth arise between successive transverse connectives. A large trachea is given off from the base of the eighth dorsal anastomosis to supply the posterior part of the 'aorta'.

Ventral ganglionic tracheae (*v.g.*). These arise from the lateral longitudinal trunks immediately posterior to where they anastomose with the third to the seventh transverse connectives. As the ganglia are concentrated in the thoracic region the ganglionic tracheae are similarly displaced.

Ventral anastomoses (*v.a.*). These correspond, in the thoracic segments, to the ventral ganglionic tracheae. In typical dipterous larvae ganglionic tracheae run from each anastomosis to the appropriate ganglion. These are absent in *Gasterophilus*. The first ventral anastomosis is also abnormal in that it originates from the lateral longitudinal trunk between the origin of the second ventral anastomosis and the union of the lateral and dorsal longitudinal trunks. The third ventral anastomosis arises just posterior to the anastomosis between the lateral longitudinal trunk and the first transverse connective.

Ventral cervical tracheae (*v.c.*). These are large tracheae given off from the dorsal longitudinal trunks a short distance posterior to the termination of the lateral longitudinal trunk. In *Gasterophilus*, unlike other dipterous larvae, the ventral cervical does not give off the first ventral anastomosis.

Dorsal cervical tracheae (*d.c.*). These arise from the dorsal longitudinal trunks just posterior to the origin of the ventral cervicals. After giving off a branch that unites with its fellow to form the dorsal cervical anastomosis (*d.c.a.*) and then runs to the posteriorly displaced supraoesophageal ganglion, the dorsal cervical continues a short way to supply the posterior parts of the buccopharyngeal apparatus.

From the above account it can be seen that there are certain obvious differences from the basic plan produced by Whitten. Thus, the presence of only seven transverse connectives (instead of eight), eight dorsal anastomoses (instead of ten) and deficiences in the number of ventral ganglionic tracheae suggest the absence of a complete tracheal metamere. This is without precedent and, indeed, Whitten suggests that perhaps Imms (1942) is mistaken when he figures only seven transverse connectives and nine dorsal anastomoses for *Braula coeca*. Secondly, there is the presence of the four pairs of large conical tracheae arising directly from the spiracular chamber at the same level as the dorsal longitudinal trunk. Thirdly, there is the abnormal distribution of the tracheae arising from the anterior terminations of the dorsal and lateral longitudinal trunks. Finally, there is an irregular loss of certain tracheae. Thus, there are no ganglionic branches from the three ventral anastomoses and the second transverse connective lacks a ventral ganglionic and a visceral trachea. The loss of the ganglionic branches is surprising as Whitten says they were invariably present in all the forms she studied. The visceral tracheae seem to be more variable in their number and distribution.

The homologies of the conical tracheae of the tracheal organ. From a study of the tracheal system of first instar larvae the first two problems were seen to be connected. As already mentioned the tracheal system is fundamentally the same as in the succeeding instars. The posterior tracheae, although to a certain extent

obscured by the tracheolation of the developing tracheal organ, are less modified and have a more normal arrangement. As there is no common spiracular chamber there is a well developed last dorsal anastomosis uniting the dorsal longitudinal trunk of each side shortly after they arise from the relatively long felt-chambers of the separate spiracles (Figs. 1, 4). Two of the tracheae (3, 4 *c.t.*) of the developing tracheal organ and the large posterior visceral trachea arise from a short, stout, ventral trunk which leaves the dorsal longitudinal trunk in the region of the last dorsal anastomosis (Fig. 4). The remaining two tracheae of each side arise a little more anteriorly, one laterally (2 *c.t.*) and the other dorsally (1 *c.t.*). The lateral longitudinal trunk (*l.l.t.*), from its anastomosis with the seventh transverse connective (7 *tr.c.*), runs posteriorly into a mass of tracheoles. By crushing the larva in gum-arabic under a cover-slip it was possible, by a little manipulation, to trace the connexions further. Thus, the first anastomosis of the lateral longitudinal trunk after the seventh transverse connective is with the distal tracheae of the dorsolateral conical trachea (2 *c.t.*). A continuation of the lateral longitudinal trunk arises a short distance proximal to its anastomosis with the second conical trachea and runs back to join the short trachea that gives off the third and fourth conical tracheae (3, 4 *c.t.*) and the posterior visceral trachea (Fig. 4). The dorsal conical trachea (1 *c.t.*) has no anastomosis with other tracheae.

The two anastomoses with the lateral longitudinal trunk and the point of origin of the posterior visceral trachea provide conclusive evidence of the homologies of the conical tracheae. The continuation, posteriorly, of the lateral longitudinal trunk after its anastomosis with the second conical trachea indicates that this lateral conical trachea represents a modified eighth transverse connective. This tracheal metamere lacks a visceral and a ventral ganglionic trachea but the first conical trachea, from its dorsal position, probably represents the dorsal anastomosis. This cannot be confirmed as there is no anastomosis between the two dorsal conical tracheae. The two conical tracheae given off by the large ventral trachea can be regarded as specialized tracheae given off by the terminal section of the lateral longitudinal trunk. That the large ventral trachea represents the terminal section of the lateral longitudinal trunk is confirmed by the point of origin of the posterior visceral trachea as this is always given off by the lateral longitudinal trunk just before its union with the dorsal longitudinal trunk. Furthermore, the terminal regions of the lateral longitudinal trunk, in all the forms studied in this investigation, give off numbers of tracheae to supply the body musculature and in the first instar larvae of *Gasterophilus* these arise from the ventral trunk. It is also known that specialized structures at the posterior end of the body such as the anal papillae of *Aëdes* and *Eristalis* and the posterior respiratory processes of *Cryptochaetum striatum* (as suggested by Thorpe's (1941) description) are supplied by large adventitious tracheae similar to the third and fourth conical tracheae.

In the succeeding instars, with a fully developed tracheal organ, the specialization of the tracheal system is complete. From the condition in the second instar (Fig. 6) it can be seen that the second conical trachea (2 *c.t.*) represents the eighth transverse connective. The continuation of the lateral longitudinal trunk (*l.l.t.*) to

the trachea running from the base of the fourth conical trachea (4 *c.t.*) indicates that the latter and the third conical trachea (3 *c.t.*), which gives off a similar trachea from its base, represent the tracheae originating from the ventral trachea of the first instar. The dorsal conical tracheae (1 *c.t.*) must represent the dorsal anastomosis and this is supported by its similarity to the second conical trachea in sending only a small tuft of tracheoles from its tip to the body musculature.

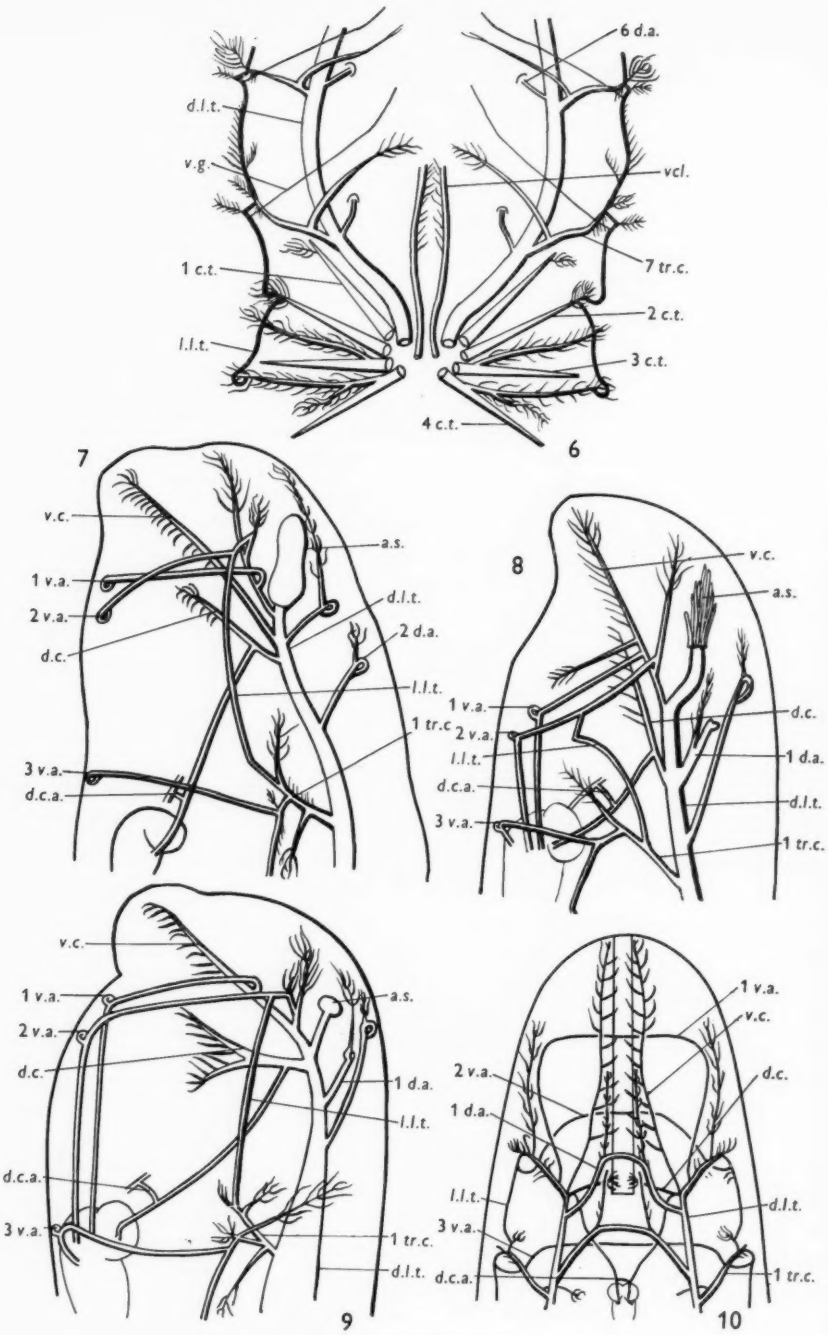
The arrangement in the second and third instars suggests therefore that the dorsal longitudinal trunk of each side is antero-posteriorly compressed between the levels of the spiracles and the eighth transverse connective into the region of the tenth dorsal anastomosis. The lumen of the dorsal anastomosis then becomes the common spiracular chamber; with the openings of the conical tracheae, the dorsal longitudinal trunks and the posterior visceral tracheae on the anterior face.

Distribution of the anterior tracheae. These tracheae, in their detailed distribution, differ from those in the plan of *Calliphora* given by Whitten (1955). In *Gasterophilus* (Figs. 7, 10) the lateral longitudinal trunk terminates, after giving off the second ventral anastomosis (2 *v.a.*), at the distal end of a short branch from a large trachea which arises from the anterior end of the dorsal longitudinal trunk and supplies the dorsolateral musculature of the anterior regions. This large trachea also gives off the first ventral anastomosis (1 *v.a.*) from a point just proximal to the origin of the branch which receives the lateral longitudinal trunk. Just posterior to the origin of this large trachea there arises another large trachea, the ventral cervical (*v.c.*), which runs forward to the anterior of the buccopharynx and the antennary papilla. The dorsal cervical arises posteriorly to the ventral cervical and supplies only the posterior part of the buccopharynx; the dorsal cervical anastomosis and the first dorsal anastomosis, with its forwardly directed branches, are normal.

As it was difficult to compare this distribution with that of Whitten (1955), a study of the homologies of these anterior tracheae was made in larvae of the following Cyclorrhapha: *Drosophila* sp., *Eristalis tenax* and a further study of *Calliphora erythrocephala*. It was also necessary to examine examples of the Nematocera; these were *Scatopse notata* and *Aëdes aegypti*. In all these larvae most of the tracheal system adhered very closely to the basic plan of Whitten, but there were significant differences in the arrangement of the anterior tracheae and, therefore, these will be described in some detail.

The anterior tracheal system of larvae of Drosophila (Fig. 8). These tracheae differ in their distribution from those of *Gasterophilus* and *Calliphora* (Whitten, 1955). Thus, the lateral longitudinal trunk (*l.l.t.*) becomes much stouter after giving off first the second ventral anastomosis (2 *v.a.*) and then a large trachea to the antero-lateral musculature. It then unites with the ventral cervical (*v.c.*) and continues as a well marked common trunk for a short distance before uniting with the dorsal longitudinal trunk (*d.l.t.*). As in *Gasterophilus*, the dorsal cervical (*d.c.*) does not run far forward and supplies only the posterior part of the buccopharynx, whilst the large ventral cervical supplies the anterior regions and the antennary papilla.

The anterior tracheal system of larvae of Eristalis (Fig. 9). The larval tracheal system was found to be very much as figured by Gäbler (1930). The anterior



Figs. 6–10. For legends see opposite page.

tracheae are similar in pattern to those of *Drosophila*. Thus, the lateral longitudinal trunk (*l.l.t.*) enlarges considerably shortly after it gives off the second ventral anastomosis (2 *v.a.*) and gives off a large branch to the antero-lateral musculature. It then unites with the ventral cervical (*v.c.*) to form a common trunk which joins the dorsal longitudinal trunk (*d.l.t.*) after a short distance. The ventral cervical (*v.c.*) is also large and well developed; it runs forward from the common trunk to supply the most anterior parts of the buccopharynx and the antennary papilla. The dorsal cervical (*d.c.*) is very large and divides into two main groups of tracheae. Of these one group supplies the greater part of the anterior buccopharynx and the other the posterior part. The dorsal cervical anastomosis and its ganglionic branch arise normally at the base of the dorsal cervical.

Thus, *Drosophila* and *Eristalis* have identical patterns of distribution of the anterior tracheae: the lateral longitudinal trunk and the ventral cervical join the dorsal longitudinal trunk by means of a short, but distinct, common trunk. Furthermore, as in *Gasterophilus*, the more anterior regions are supplied by the ventral cervical whilst the dorsal cervical is a relatively short, stout trachea supplying the posterior of the buccopharynx alone.

The anterior tracheal system of Calliphora (Fig. 11). The anterior tracheal system, at first examination, seemed to agree with Whitten's description. However, it was found that in the views normally seen, after injection or by compression, the tracheae tend to obscure each other. This results in the apparent uniting of all the tracheae at the one point as shown by Whitten. It was found that, in fact, the lateral longitudinal trunk (*l.l.t.*), after anastomosing with the first transverse connective (1 *tr.c.*), loops ventrally and diminishes greatly in diameter. It then runs dorsally, gives off the second ventral anastomosis (2 *v.a.*), increases slightly in size and continues dorsally to the point where it enlarges greatly and gives off a large trachea to the antero-lateral musculature and another which runs inwards and backwards to supply the large muscle which links the first and fourth segments. Close to this point there is the junction with the ventral cervical (*v.c.*) which gives rise to the common trunk which almost immediately enters the dorsal longitudinal trunk (*d.l.t.*). The ventral cervical is well developed and gives off the first ventral anastomosis (1 *v.a.*) shortly after separating from the common trunk and then runs forward to supply the anterior two-thirds of the buccopharynx and the antennary papilla. The dorsal cervical (*d.c.*) supplies only the posterior third of the buccopharynx after giving off the branch to the dorsal cervical anastomosis and the supraoesophageal ganglion. The picture given by Whitten is due to the fact that the ventral cervical arises from the inner side of the common trunk and is

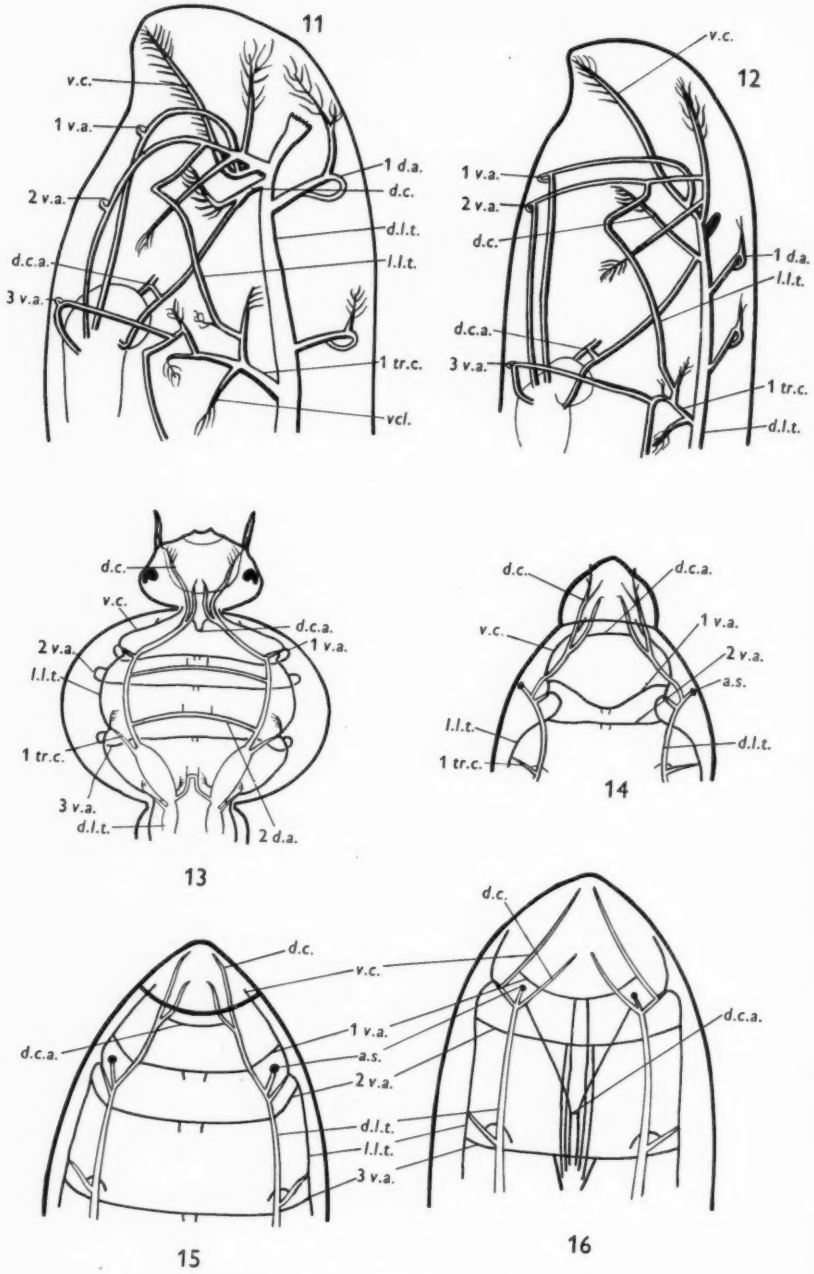
Fig. 6. The posterior tracheae of the second instar larva (and as is postulated for the third instar) of *Gasterophilus* showing the relationships of the conical tracheae. Ventral view.

Fig. 7. The anterior tracheal system of the third instar larva of *Gasterophilus*. Lateral view.

Fig. 8. The anterior tracheal system of the larva of *Drosophila*. Lateral view.

Fig. 9. The anterior tracheal system of the larva of *Eristalis*. Lateral view.

Fig. 10. The anterior tracheae of the first instar larva of *Gasterophilus*. Dorsal view.



Figs. 11–16. For legends see opposite page.

obscured, in lateral view, by the base of the lateral longitudinal trunk. As the ventral cervical continues it, in turn, overlies the dorsal cervical and in fact the trachea labelled dorsal cervical in Whitten's plan must be the ventral cervical. The ventral cervical of her plan is the antero-lateral branch of the lateral longitudinal trunk and appears to be giving off the first ventral anastomosis as that trachea is really passing under the lateral longitudinal trunk before joining the true ventral cervical. In the first instar (Fig. 12) the position is a little clearer and fully confirms the above interpretation.

In order to investigate the validity, as a general observation, of the occurrence of a common trunk linking the lateral longitudinal and the ventral cervical tracheae with the dorsal longitudinal trunk a study was made of two nematocerous larvae.

The anterior tracheal system of Aëdes (Fig. 13). The dorsal longitudinal trunk is greatly dilated for most of its length, with slight constrictions at the origin of each transverse connective. In the larval thorax it becomes reduced to more normal proportions at the level of the first transverse connective. The lateral longitudinal trunk (*l.l.t.*) runs forward from the level of the first transverse connective (1 *tr.c.*), gives off the second ventral anastomosis (2 *v.a.*) and then unites with the ventral cervical (*v.c.*) to form a distinct common trunk before joining the dorsal longitudinal trunk (*d.l.t.*). The ventral cervical is a small trachea which, after giving off the first ventral anastomosis (1 *v.a.*), does not appear to run far into the head capsule. The dorsal cervical (*d.c.*) is a large trachea which, after giving off the branch to the dorsal cervical anastomosis and the supraoesophageal ganglion, continues to supply most of the head including the antennae.

The anterior tracheal system of Scatopse (Fig. 14). The lateral longitudinal trunk (*l.l.t.*) after anastomosing with the first transverse connective (1 *tr.c.*) runs forward, gives off the second ventral anastomosis (2 *v.a.*) and then unites with the ventral cervical (*v.c.*). The common trunk runs for a short distance before terminating in the dorsal longitudinal trunk (*d.l.t.*). The ventral cervical is a small trachea which runs forward into the head capsule after giving off the first ventral anastomosis (1 v.a.). The dorsal cervical (*d.c.*) is a very large trachea which, after giving off the trachea to the supraoesophageal ganglion and the dorsal cervical anastomosis, enters the head capsule and breaks up into a number of tracheae supplying the majority of the tissues including the larval antennae.

Fig. 11. The anterior tracheal system of the third instar larva of *Calliphora*. Lateral view.

Fig. 12. The anterior tracheal system of the first instar larva of *Calliphora*. Lateral view.

Fig. 13. The anterior tracheal system of larvae of *Aëdes*. Dorsal view.

Fig. 14. The anterior tracheal system of the larva of *Scatopse*. Dorsal view.

Fig. 15. Diagram showing the typical distribution of the anterior tracheae in the larvae of *Nematocera*. Dorsal view.

Fig. 16. Diagram showing the typical distribution of the anterior tracheae in the larvae of *Cyclorrhapha*. Dorsal view.

Thus, at least in the two forms studied, the termination of the lateral longitudinal trunk and the ventral cervical with the dorsal longitudinal trunk is by means of a common trunk in the Nematocera as well as in the Cyclorrhapha.

DISCUSSION

The evidence produced fully bears out the views of Whitten (1955) on the remarkable constancy of the tracheal system of larval Diptera. This was emphasized by the demonstration of the derivation of the tracheae of the tracheal organ of larvae of *Gasterophilus* from a complete tracheal metamere together with two adventitious tracheae from the lateral longitudinal trunk. In some other Diptera specialized tracheae are developed to a lesser degree from the posterior section of the lateral longitudinal trunk in, for example, *Aëdes* (to the anal gills), *Eristalis* (to the anal gills and the siphon) and perhaps in *Cryptochaetum*, but, in *Gasterophilus*, the production of specialized tracheae from this section in parallel with a similar modification of an entire tracheal metamere would seem to be unique.

The formation of a common spiracular chamber and the large size of the spiracular plates, together with the very large openings into the conical tracheae, can probably all be correlated with the need for very efficient and rapid tracheal ventilation. This is necessary to enable the functionally metapneustic larva to take advantage, by a complete oxygenation of the haemoglobin of the tracheal organ, of any air pockets occurring in the horse's stomach. In this connexion it was noted that many larvae maintained *in vitro* show, by the dark purple colouring of the deoxygenated haemoglobin of the tracheal organ, that the oxygen tension in the tracheae is very low. If the lips of the spiracular pouch are opened by forceps the tracheal organ flushes bright scarlet and appears fully oxygenated within 2 sec. Since this oxygen will support the larva only for a maximum period of 4 min., and is only given up at very low oxygen tensions, it is probably very much a last reserve. Although the animal is capable of surviving long periods of anaerobiosis this is probably a relatively inefficient process. Hence the desirability of quickly replenishing the basic oxygen reserve has led to these specializations in the morphology of the tracheal system.

Although the plans of Whitten (1955) for *Aphiochaeta* and *Calliphora* differ from each other and from the plan for the Nematocera in the detailed arrangement of the tracheae supplying the anterior segments, the present investigation has shown there is no great difference. In her plan for *Aphiochaeta* Whitten clearly shows the lateral longitudinal trunk giving off, firstly, the second ventral anastomosis, secondly, a lateral trachea and then joining with a large ventral cervical to form a common trunk that shortly unites with the dorsal longitudinal trunk. The Nematocera were also shown to have the typical arrangement of the anterior tracheae. This arrangement has been shown to be present in *Calliphora* and all the other Cyclorrhapha studied with the exception of *Gasterophilus*, in which the arrangement is definitely abnormal in that the lateral longitudinal trunk and the ventral cervical unite separately with the dorsal longitudinal trunk and in that the first ventral anastomosis arises from the lateral longitudinal trunk. There

seems to be no obvious explanation for this anomalous arrangement which exists in all stages of larval development.

One feature of the change from the nematocerous condition possessing a head capsule and the cylorrhaphous where the larval head segments are invaginated into the mouth is the change in relative size of the ventral and dorsal cervicals (Figs. 15, 16). In the nematocerous condition the dorsal cervical seems to be the more important, supplying most of the tissues of the head capsule while the ventral cervical is very small, in some cases terminating almost immediately it enters the head capsule. This condition is perhaps not invariable as in some of Whitten's figures the ventral cervical is shown as being the same size as the dorsal cervical and therefore perhaps supplying an equal share of the head capsule. It does, however, indicate a tendency for the ventral cervical to be of less importance and to penetrate only a short distance into the head capsule. Within the Cyclorrhapha, however, the dorsal cervical is restricted to the posterior regions of the buccopharyngeal apparatus, while the branch to the supraoesophageal ganglion is reflected backwards with the posterior displacement of the ganglion. Moreover, the ventral cervical has become greatly enlarged and supplies the more anterior regions of the buccopharynx and the anterior regions of the new larval 'head'. These regions in the nematocerous condition are those of the extreme posterior of the head capsule and the anterior face of the first thoracic segment, and it is here that the ventral cervical appears to terminate in many Nematocera. This indicates that the remarkable stability of the tracheal system towards anatomical specializations is due to a marked degree of specificity towards the embryological segments.

SUMMARY

1. The tracheal systems of first, second and third instar larvae of *Gasterophilus intestinalis* are described.

2. The homologies of the conical tracheae of the tracheal organ are demonstrated and explain the apparent disappearance of a complete tracheal metamere.

3. Anomalies in the tracheal system of the anterior segments of *Gasterophilus* led to an investigation of the tracheal systems of *Drosophila*, *Eristalis*, *Calliphora*, *Scatopse* and *Aëdes*.

4. The anterior tracheal system of larval Diptera has been re-interpreted; that of the Cyclorrhapha can be derived from that of the Nematocera without the formation of new tracheae other than a large trachea from the lateral longitudinal trunk to the antero-lateral musculature.

Thanks are due to Dr P. Tate for his advice and encouragement throughout this investigation.

REFERENCES

Carpenter, G. H. & Pollard, F. J. S. (1918). The presence of lateral spiracles in the larva of *Hypoderma*. *Proc. R. Irish Acad.* **34**, 73–84.

Dinulescu, G. (1932). Récherches sur la biologie des gastrophiles. *Ann. Sci. Nat. Zool.* (10), **15**, 1–184.

Enderlein, G. (1899). Die respirations Organe der Gastriden. *S.B. Akad. Wiss. Wien*, **108**, 235–303.

GÄBLER, H. (1930). Die postembryonale Tracheensystems von *Eristalis tenax* L. *Z. Morph. Ökol. Tiere*, **19**, 427–92.

IMMS, A. D. (1942). On *Braula coeca* Nitsch and its affinities. *Parasitology*, **34**, 88–100.

KEILIN, D. (1944). Respiratory systems and respiratory adaptations in larvae and pupae of Diptera. *Parasitology*, **36**, 1–66.

KEILIN, D. & WANG, Y. L. (1946). Haemoglobin of *Gastrophilus* larvae. Purification and properties. *Biochem. J.* **40**, 855–66.

KEMNITZ, G. A. VON (1916). Untersuchungen über den Stoffbestand und Stoffwechsel der Larven von *Gastrophilus equi* (Clark), nebst bemerkungen über den Stoffbestand der Larven von *Chironomus* (Spec?) L. (Physiologischer Teil.). *Z. Biol.* **67**, 129–244.

LANGERON, M. (1949). *Précis de microscopie*. Paris: Masson et Cie.

LEVENBOOK, L. (1951). The effect of carbon dioxide and certain respiratory inhibitors on the respiration of larvae of the horse bot-fly (*Gasterophilus intestinalis* De Geer). *J. Exp. Biol.* **28**, 181–202.

NIELSEN, J. C. (1909). *Ent. Medd.* IV, 1 (cited in Keilin, 1944).

PANTEL, J. (1898). *Cellule*, **15**, 1 (cited in Keilin, 1944).

PORTIER, P. (1911). Récherches physiologiques sur des insectes aquatiques. *Arch. Zool. exp. gén.* **8**, 89–379.

PRENANT, A. (1899). Terminaison intracellulaire et réellement cytoplasmique des trachées chez la larve de l'Oestre du cheval. *C.R. Soc. Biol., Paris*, 11 série, **1**, 507–10.

PRENANT, A. (1900). Notes cytologiques. Cellules tracheales des Oestres. *Arch. Anat. micr.* **3**, 293–336.

TAPPEINER, H. (1883). Die Gase des Verdauungschlauches der Pflanzenpresser. *Z. Biol.* **19**, 228–79.

THORPE, W. H. (1941). The biology of *Cryptochaetum* (Diptera) and *Eupelmus* (Hymenoptera) parasites of *Aspidoproctus* (Coccidae) in East Africa. *Parasitology*, **33**, 149–68.

WHITTEN, J. M. (1955). A comparative morphological study of the tracheal system of larval Diptera. Part I. *Quart. J. Micr. Sci.* **96**, 257–78.

WIGGLESWORTH, V. B. (1950). A new method for injecting the tracheae and tracheoles of insects. *Quart. J. Micr. Sci.* **91**, 217–24.

LIST OF ABBREVIATIONS

a.s. anterior thoracic spiracle
a.t. trachea supplying the posterior region of the aorta
c.t. conical tracheae of the tracheal organ
d.a. dorsal anastomosis
d.c. dorsal cervical
d.c.a. dorsal cervical anastomosis
d.l. dorsal lip of the spiracular pouch
d.l.t. dorsal longitudinal tracheal trunk
l.l.t. lateral longitudinal tracheal trunk
p.s. postabdominal spiracles
t.b. tracheal body
tr.c. transverse connective
v.a. ventral anastomosis
v.c. ventral cervical trachea
vcl. visceral trachea
v.g. ventral ganglionic trachea

Parasitology (1960), **50**, 497–499
Printed in Great Britain

Invasion of the egg masses of the mollusc *Bulinus* (*Physopsis*) *globosus*, by larval chironomids

By G. PRINGLE
East African Institute of Malaria and Vector Borne Diseases, Amani, Tanganyika

(*Received* 29 *February* 1960)

Small colonies of the freshwater snail, *Bulinus* (*Physopsis*) *globosus*, are maintained in the Institute laboratories at Amani, Tanganyika. The water in the rearing bowls is replenished regularly from Amani lake, where this species of snail is common.

In January 1959 it was noticed that an egg mass of *B.* (*Physopsis*) *globosus* in a laboratory rearing bowl contained a chironomid larva. The contents of the egg mass had been partly eaten and the larva was in the empty section of it. During the next 2 days this larva entered the remaining capsules and cleared them of their embryos. The chironomid larva then left the original egg mass and invaded another one, which was attached to the bowl a little distance away. The larva pupated in the latter egg mass and an adult chironomid midge, identified as *Polypedilum* (*Polypedilum*) *kibatiense* Gtgh, emerged.

It was decided that a limited investigation should be carried out to find out whether this predation occurred in nature and, if this proved to be so, whether the relationship between predator and host was obligatory or casual.

At intervals between February and May, 1959, examinations were made of egg masses of *B. globosus* attached to lily pads in Amani lake. In the course of these visits 123 of these egg masses were scrutinized *in situ*, and it was found that eleven of them were, at the time of examination, occupied by active chironomid larvae. A larger (but uncounted) number of the egg masses of *Lymnea natalensis* was observed in the same habitat, but there was no indication that this snail was suffering similar predation.

An effort was made to rear the larvae collected from the egg masses of *Bulinus globosus* in Amani lake. Nine adults subsequently hatched and were identified: seven were *Polypedilum kibatiense*, one was *Polypedilum* (*Pentapedilum*) *anale* Freeman, and one was *Chironomus* (*Cryptochironomus*) *acutus* Gtgh.

To obtain information on the character of the relationship between predator and host, two further small collections were made from Amani lake, one of chironomid larvae collected from an egg mass of *Bulinus globosus*, the other of larvae from adjacent lily pads, which were not attacking snail egg masses at the time of collection. Half the larvae in each of these two groups were fed in the laboratory on egg masses of *B. globosus* and the others were fed on pond detritus only. The results of this experiment are summarized in Table 1.

The voracity of chironomid larvae may be gauged from the following experiment.

A newly collected specimen was encouraged to feed on egg masses of *B. globosus* in a small dish in the laboratory. Between the 28 May, when it was collected, and the 4 June, when it pupated, the larva consumed sixty-one snail embryos in all stages of development from the trochosphere to the veliger. It would seem that a chironomid larva might destroy as many as four complete egg masses during the course of its development.

Table 1

	Site of collection	Subsequently fed on	Days to emerge	Species
1	Egg mass	Egg masses	1	*Polypedilum* (s.str.) sp.indet.
2	Egg mass	Egg masses	2	*P. anale*
3	Egg mass	Egg masses	2	*P. anale*
4	Egg mass	Egg masses	3	*P. kibatiense*
5	Egg mass	Detritus	4	*P. kibatiense*
6	Egg mass	Detritus	14	*P. kibatiense*
7	Egg mass	Detritus	9	*P. kibatiense*
8	Egg mass	Detritus	Died in the pupal stage	—
9	Lily pad	Egg masses	3	*P. kibatiense*
10	Lily pad	Egg masses	4	*P. kibatiense*
11	Lily pad	Egg masses	5	*P. kibatiense*
12	Lily pad	Egg masses	10	*P. kibatiense*
13	Lily pad	Detritus	2	*P. kibatiense*
14	Lily pad	Detritus	3	*P. anale*
15	Lily pad	Detritus	Died in the larval stage	—
16	Lily pad	Detritus	Died in the larval stage	—

DISCUSSION

The larvae of several species of chironomid appear to act as occasional predators of the egg masses of *Bulinus* (*Physopsis*) *globosus* in Amani lake. It would, furthermore, seem that embryonic snail tissue is eagerly sought by these insect larvae and is a highly suitable diet for them.

The observation made that the chironomid larvae select the egg masses of *B.* (*Physopsis*) *globosus*, and do not feed on those of *Lymnea*, recalls observations made by Jutting (1938). Jutting noted that certain chironomid larvae construct their tunnels on the shell of the pulmonate snail *Physa fontinalis*, and in some instances invade the pulmonary cavity of this snail. Although in this association the *Physa* appeared to suffer inconvenience only, Jutting (1938) also cited earlier papers which record that chironomid larvae may trap *Physa*, immobilize it and then feed on it. During the observations here recorded, however, it was noted that *Lymnea* snails, although they were present in the same pond at the same time, were ignored by the chironomid larvae.

The present notes on the feeding behaviour of chironomid larvae at Amani afford a possible parallel with the previously reported inquilism and predation of *Physa* by chironomid larvae. There is here perhaps a hint of some fundamental biological relationship between these larvae and certain physid and planorbid snails.

Bulinus (*Physopsis*) *globosus* is a vector of human schistosomiasis *Schistosoma haematobium* in Africa. Assessment of the effect of this type of predation on *B. globosus* populations is therefore desirable. No attempt has yet been made to do this. It may be stated, however, that the density of adult *B. globosus* in Amani lake is high during much of the year and that it remained so while the observations here recorded were made in 1959.

SUMMARY

1. It has been observed that the larvae of four species of chironomid midges invade and destroy the contents of the egg masses of *Bulinus* (*Physopsis*) *globosus* in Amani lake, Tanganyika.

2. There was no indication that this type of predation exercised any restraint on the density of adult snails.

The author is indebted to Dr Paul Freeman of the British Museum for his generous help in the identification of all chironomid spp. and to Mr E. Hemingway and other members of the Institute staff for their participation in this work.

REFERENCE

JUTTING, T. VAN BENTHEM (1938). A freshwater Pulmonate (*Physa fontinalis*) inhabited by the larva of a non-biting midge (*Tendipes* (*Parachironomus*) *varus* Gtgh.). *Arch. Hydrobiol.* (*Plankt.*) **32**, 693.

Parasitology (1960), **50**, 501–508
With 2 text-figures
Printed in Great Britain

Lipids in different stages of the life cycles of malaria parasites and some other sporozoa

By B. DASGUPTA

*Department of Parasitology, London School of Hygiene and Tropical Medicine**

(*Received* 14 *March* 1960)

INTRODUCTION

Akashi (1930) used Nile Blue as a supravital stain for studying the erythrocytic stages of different species of the malaria parasites. His report indicated that these parasites consisted of two portions: a larger unstained portion, and a smaller stained portion. In the present study lipids have been studied in three species of malaria parasites (*Plasmodium gallinaceum*, *P. cynomolgi* and *Hepatocystis kochi*) in the different stages of their life cycles, by using cytochemical staining methods, including the Nile Blue technique, and the results obtained are compared with those of similar studies made on some other Sporozoa (*Hepatozoon sciuri*, *H. balfouri* and *Eimeria stiedae*) in the available stages of their life cycles.

Thélohan, as early as in 1894, reported an increase of fat globules in the oocysts of certain species of coccidia, during their degeneration. Gurwitsch (1927) reported the occurrence of materials stained with Sudan III in the gametocytes and the schizonts, and also occasionally in the oocysts, of *E. stiedae*. According to Cazet, Abbate & Duchini (1932) the advanced stages of the parasite designated by them as *Coccidium cuniculi*, displayed a loss of perinuclear distribution of fat, which is characteristic of the younger forms, and an invasion of the cytoplasm by the fatty materials. In the present investigation the different stages of sporogony in the life cycle of *Eimeria stiedae* have been cytochemically examined for the presence of lipids.

MATERIAL AND METHODS

Plasmodium gallinaceum was studied in the laboratory-bred mosquitoes (*Aëdes aegypti* var. *queenslandensis*) and in the experimentally infected 1-week-old chick (*Gallus domesticus*). The erythrocytic stages and the male gametes were studied in blood smears. *Plasmodium cynomolgi* was studied in the laboratory-bred mosquitoes (*Anopheles maculipennis* var. *atroparvous*) and in experimentally infected monkeys (*Macacus rhesus*). The sporogony stages were obtained in smears of the infected stomachs and salivary glands; the pre-erythrocytic schizonts were studied in frozen sections of pieces of liver of infected monkeys obtained by biopsy, while the erythrocytic stages and the male gametes were studied in the blood smears.

The gametocytes of *Hepatocystis kochi* were studied in blood smears obtained from the naturally infected monkey (*Cercopithecus aethiops*).

The gametocytes of *Hepatozoon sciuri* and those of *H. balfouri* were studied in

* Present address: Biological Laboratory, Darjeeling Government College, Darjeeling, India.

blood smears obtained from their respective vertebrate hosts (*Sciurus carolinensis* and *Jaculus jaculus*).

The sporogony stages of *Eimeria stiedae* were studied by breaking the hard wall of the oocysts obtained from the experimentally infected rabbits (*Oryctolagus cuniculus*), by pressure.

The blood smears were fixed in formol-saline for 1 min. only; the salivary glands of the mosquitoes were fixed in the same fixative for 1–2 hr.; the stomachs of the infected mosquitoes were fixed in formol-saline for 5–6 hr.; pieces of the liver from the infected monkey obtained by biopsy were fixed in formol-calcium for 18 hr. for frozen sectioning, while the pieces of brain, heart and kidney of the infected chicks were fixed in formol-saline and preserved in the same fluid for a week before preparing the frozen sections; the sporogony stages of *Eimeria stiedae* were fixed in formol-saline for 1 hr. only.

Materials fixed in calcium-formalin and formol-saline were used in smears and in frozen sections, and stained with Sudan Black. Staining for 30 min. at room temperature was found to yield satisfactory result; 1 % aqueous solution of Neutral Red was sometimes used as counterstain. Saturated alcoholic solution of Fettrot 7B was used for 20–30 min., and Ehrlich's haematoxylin was used as counterstain; in some cases Sudan III and Sudan IV stains were also used. Nile Blue was used to detect neutral and acidic lipids in smears and also in frozen sections. Acid haematein method for phospholipids was employed both with and without mordanting in dichromate-calcium. Copper phthalocyanin method for the detection of phospholipids was performed (Pearse, 1955) using Luxol Fast Blue as the reacting dye. Pyridine extraction procedure was followed in all cases.

OBSERVATIONS

The following observations were made.

Plasmodium gallinaceum (Fig. 1)

The majority of the oocysts of all ages were found to contain reacting material in the cytoplasm in the form of globular masses when Sudan Black was used: they were absent only in a small number of oocysts. The Sudanophil globular material sometimes appeared to have an unstained central core. From the 6th day of infection onwards a diffuse black reaction was noticed in the cytoplasm, in addition to the globular reacting material referred to above. This diffuse reaction reached its maximum from the 7th to the 9th days of infection, when the entire oocysts appeared almost black. This diffuse Sudanophil material did not, however, occur simultaneously in all the oocysts of the same age; as a result, it was often seen that the oocysts which stained almost black lay alongside the faintly stained or the unstained ones. In the mature oocysts the Sudanophil material appeared scattered here and there in between the sporozoites.

With Sudan IV stain, reacting globular material was noticed in the oocysts, but the result was poor in comparison to that seen with Sudan Black method. With Fettrot method, reacting material in the form of globular masses was seen

in the oocysts. With Nile Blue method, violet-coloured globules were seen in the oocysts.

With acid haematein technique the result obtained was similar to that seen with Sudan Black stain. The reacting globular masses occurring in the cytoplasm often had an unstained central core. Between the 6th and 9th days of infection some

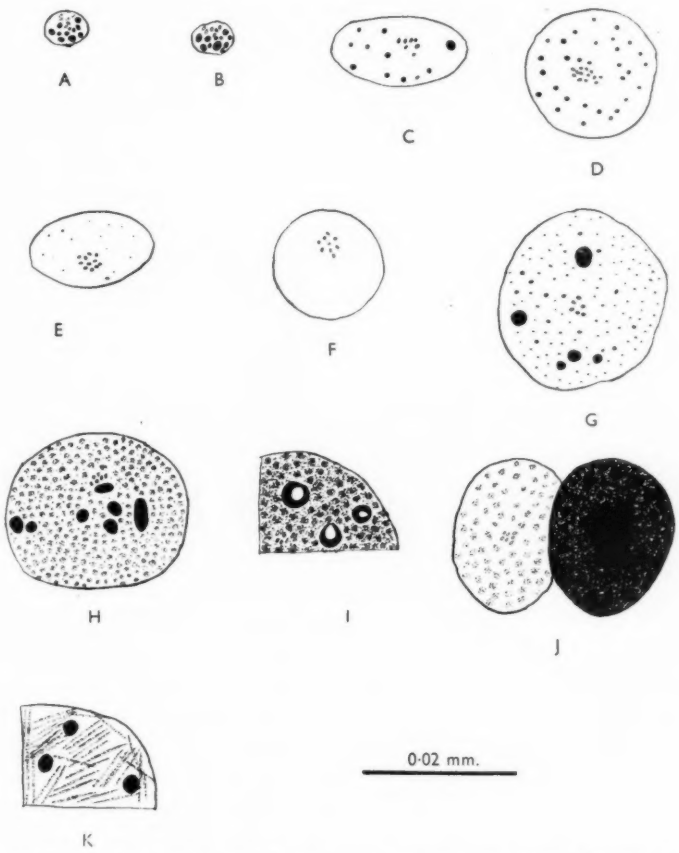


Fig. 1. The oocysts of *Plasmodium gallinaceum*, stained with Sudan Black. A, B, 3-day-old oocysts; C, a 4-day-old oocyst; D–F, 5-day-old oocysts; G, a 6-day-old oocyst; H, a 7-day-old oocyst; I, an 8-day-old oocyst; J, two 8-day-old oocysts; K, a 9-day-old oocyst, showing the reacting globules lying in between the sporozoites.

oocysts took up deep blue colour throughout the cell body; while during the same period some other oocysts took up less intense colour or remained unstained.

With copper phthalocyanin technique the oocysts exhibited various types of colour reactions. In the early stages some small oocysts remained unstained, while others were pink in colour. The later forms were deep blue and purple in colour, and the mature forms which contained sporozoites were again colourless.

It appeared that the reacting substance gradually accumulated in the oocysts, reached its maximum concentration between the 6th and 9th days of infection, and disappeared afterwards when the sporozoites were ready to be liberated. It was also seen in the material stained by copper phthalocyanin method that purple-

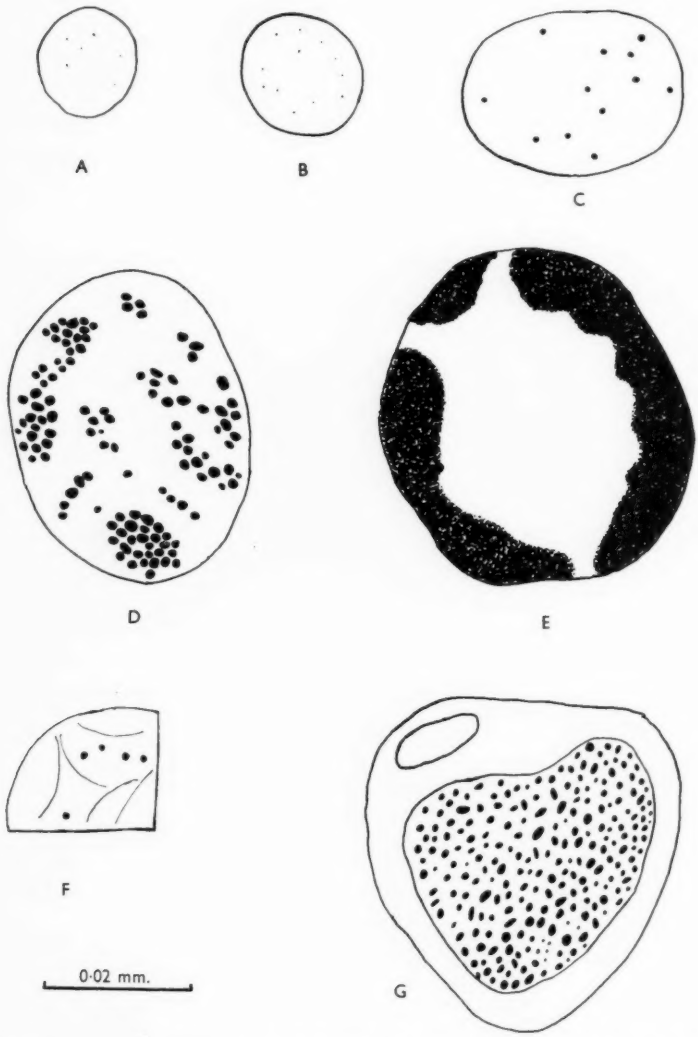


Fig. 2. *Plasmodium cynomolgi*. A, a 5-day-old oocyst stained with Sudan Black; B, a 6-day-old oocyst stained with Sudan Black; C, a 7-day-old oocyst stained with Sudan Black; D, an 8-day-old oocyst stained with Sudan Black; E, an 8-day-old oocyst showing the Sudan Black-stained material in the peripheral region; F, a 10-day-old oocyst stained with Sudan Black; G, a pre-erythrocytic schizont showing Fettrot-stained material.

coloured oocysts often lay alongside the unstained oocysts. The oocysts extracted with pyridine failed to stain with any of the above methods. Apart from some minute Sudan Black-stained granules, no other reaction for lipids was noticed in the sporozoites. The exo-erythrocytic schizonts appeared to contain some Sudan Black-stained material. The parasites in the blood smears did not stain with any of the above methods.

Plasmodium cynomolgi (Fig. 2)

With Sudan Black method the early oocysts all remained unstained. In the 5th and 6th days of infection minute Sudan Black-staining granules appeared in the oocysts. From the 7th day of infection onwards, Sudan Black-positive globules occurred in the oocysts, while a diffuse black reaction occurred on their periphery on the 8th day of infection. In the mature oocysts the reacting globules appeared scattered here and there

With Fettrot method, red-coloured globules were noticed in the oocysts; with Nile Blue method, violet-coloured globules were noticed in the oocysts; with copper phthalocyanin method, various types of colour reactions occurred, as noticed in *Plasmodium gallinaceum*. The reacting material appeared to reach a maximum on the 8th day of infection, but vanished entirely in the mature oocysts. It was noticed that not all the oocysts stained with equal intensity: oocysts lying side by side exhibited dissimilar patterns of reactions. The oocysts extracted with pyridine failed to stain with any of the methods described above. The sporozoites exhibited negative reaction.

In the 6-day-old pre-erythrocytic schizonts minute Sudan Black-positive granules were noticed. In the 8-day-old pre-erythrocytic schizonts such granules were larger in size, and occurred in abundance. The reacting substance appeared undiminished in the sections stained with Feulgen's technique and subsequently treated with Sudan Black. With Sudan IV stain a faint red tinge was noticed in the schizonts, but the reaction was not convincing. When stained with Fettrot the reacting material was found to occur in abundance in the cytoplasm of the schizonts. The reacting substance was more abundant in the 8-day-old pre-erythrocytic schizont than in the 6-day-old ones. Negative result was obtained with copper phthalocyanin method. The pre-erythrocytic schizonts extracted with pyridine, and the parasites in blood smears failed to stain with any of the above methods.

Hepatocystis kochi

Negative result was obtained in the gametocytes.

Hepatozoon sciuri

An intense reaction was obtained throughout the cytoplasm of the gametocytes, when Sudan Black stain was used.

Hepatozoon balfouri

The entire cytoplasmic area of the gametocytes stained intensely with the Sudan Black method. The other results were negative.

Eimeria stiedae

Before the onset of sporulation a large number of Sudan Black-positive globules were noticed in the oocysts. Similar globular bodies were also noticed when Fettrot method was used. In sporulated oocysts the reacting material appeared to be absent. The sporozoites and the residual body did not stain with Sudan Black, Sudan III and the copper phthalocyanin methods.

DISCUSSION

It appears from the reports of some workers that the lipid content of Protozoa might vary from species to species. According to Cheissin (1930), *Mesnilella multispiculata* had no lipid reserve, while five other species of the same genus had many lipid globules in their cytoplasm. *Trypanosoma evansi* lacked lipid reserve (Krijgsman, 1936), while granules which contained lipids were found in *T. rotatorium* (Varga & Bacsich, 1936) and in *T. gambiense* (Gerzeli, 1955). A consideration of this problem in the light of the present study is likely to prove interesting.

The patterns of reaction for lipids in the oocysts of *Plasmodium gallinaceum* and *P. cynomolgi* were found to be broadly similar. The oocysts of both these parasites contained Sudanophil material in the form of granules which appeared to increase in size with age. These oocysts at a later stage exhibited a diffuse reaction for lipids. In the oocysts of *P. cynomolgi* this diffusely stained material tended to aggregate at the periphery, while in *P. gallinaceum* this substance was found to be distributed all over the cytoplasm.

It was interesting to notice that the erythrocytic stages of *P. gallinaceum*, *P. cynomolgi* and *Hepatocystis kochi* did not stain with the Sudan dyes, while the gametocytes of *Hepatozoon sciuri*, and *H. balfouri* infecting the blood corpuscles of the respective vertebrate hosts stained intensely with these dyes. Thus, it would appear that the *Plasmodium–Hepatocystis* group of Sporozoa differed from *Hepatozoon* with regard to the affinity towards Sudan dyes, at a period of the life when they invaded the blood corpuscles of their respective hosts. It is, however, premature at this stage to state whether the differences noted above, might be regarded as one of constant specific character.

It is common experience that, when an entire stomach of the mosquito, showing a large number of oocysts of *Plasmodium*, is stained by haematoxylin, or by some other conventional methods, some of the oocysts take up a deeper stain than the rest. Good illustrations of this are to be found in the literature. The photomicrograph of the oocysts of *P. knowlesi* provided by Hawking, Mellanby, Terry & Weber (1957) may be mentioned in this connexion. It appears that no explanation has so far been offered to account for this dissimilar staining property exhibited by the individual oocysts of the same species in the same infected mosquito stomach. It is clear that an unknown substance, which imparts this deeply staining property to the oocysts, makes its appearance more quickly in some oocysts than in the rest. In the course of the present study this substance has been found to stain characteristically for phospholipids (Sudan Black, acid haema-

tein and copper phthalocyanin methods), and this stainability was lost when the oocysts were extracted with pyridine. It would appear that this phospholipid material does not occur simultaneously in all the oocysts which result from the same blood meal, probably because some of the oocysts develop faster than the others.

SUMMARY

Lipid material occurs in the oocysts of *Plasmodium* in the form of small Sudanophil granules, which appear to increase in size with the growth of the parasite. A diffuse Sudanophil material also appears at a later stage.

Phospholipid occurs at a certain stage of the oocyst, but later disappears altogether. It appears more quickly in some oocysts than in the others, found in the same infected gut.

The pre-erythrocytic schizonts of *P. cynomolgi* are found to contain abundant lipid material.

The erythrocytic stages of *Plasmodium* and *Hepatocystis* cannot be stained with the Sudan dyes, but the gametocytes of *Hepatozoon* infecting the blood corpuscles of the vertebrate hosts are found to contain abundant Sudanophil material.

Sudanophil material can be detected in the unsporulated oocysts of *Eimeria stiedae*, but not in the sporulated ones.

The author wishes to record his thanks to Professor P. C. C. Garnham of the London School of Hygiene and Tropical Medicine for his kind interest in this work. Thanks are due to Mrs M. Vizoso of the Infestation Control Division, Ministry of Agriculture, Fisheries and Food, and to Mrs K. van der Poorten of London for the supply of squirrels infected with *Hepatozoon sciuri*. The author is indebted to Dr H. Hoogstraal of the United States Naval Medical Research Unit, Cairo, for the supply of jerboa infected with *Hepatozoon balfouri*. Some of the microslides on which this paper is based were demonstrated at the laboratory meetings of the Royal Society of Tropical Medicine and Hygiene (Dasgupta, 1958*a*, *b*).

REFERENCES

Akashi, K. (1930). The study of malaria parasites by the aid of supravital staining. *Taiwan Igakkai Zasshi*. no. 299, 4.

Cazet, A., Abbate, M. & Duchini, O. (1932). Contribución al estudio citológico e histoquimico de *Coccidium cuniculi* (Leuckart), 1879. *Arch. Soc. Biol. Montevideo*, **7**, 1878.

Cheissin, E. (1930). Morphologische und systematische Studien über *Astomata* aus dem Baikalsee. *Arch. Protistenk*, **70**, 531.

Dasgupta, B. (1958*a*). Cytochemical demonstration of lipid in the pre-erythrocytic schizonts of *P. cynomolgi* (Demonstration). *Trans. R. Soc. trop. Med. Hyg.* **52**, 9.

Dasgupta, B. (1958*b*). The staining reactions for lipids in the oocysts of *Plasmodium gallinaceum* (Demonstration). *Trans. R. Soc. trop Med. Hyg.* **52**, 296.

Gerzeli, G. (1955). Recerche istochimiche de istomorfologiche sui Tripanosomidi. (Microscopia a contrasto di fase ed interferenziale). *Riv. Parassit.* **16**, 209.

Gurwitsch, B. M. (1927). Materialien zum Studium der Struktur des Coccids *Eimeria stiedae* Lindemann bei Kaninchen. *Arch. Protistenk*, **59**, 369.

Hawking, F., Mellanby, H., Terry, R. J. & Weber, W. A. F. (1957). Transmission of *Plasmodium knowlesi* by *Anopheles stephensi*. *Trans. R. Soc. trop. Med. Hyg.* **51**, 397.

KRIJGSMAN, B. J. (1936). Vergleichend physiologische Untersuchungen über den Stoffwechsel von *Trypanosoma evansi* in Zusammenhang mit der Anpassung an das Wirtstier. *Z. vergl. Physiol.* **23**, 663.

PEARSE, A. G. E. (1955). Copper phthalocyanin as phospholipid stains. *J. Path. Bact.* **60**, 554.

THÉLOHAN, P. (1894). Nouvelles recherches sur les coccidies. *Arch. Zool. exp. gén.* **3**, 541.

VARGA, L. & BACSICH, P. (1936). Neue Beobachtungen über die Struktur des *Trypanosoma rotatorium* in Zusammenhang mit seinem Verhalten in Kultur und Gewebe. *Arb. ung. biol. ForschInst.* **10**, 269.

Parasitology (1960), **50**, 509–514
With 3 text-figures
Printed in Great Britain

Polysaccharides in the different stages of the life-cycles of certain sporozoa

By B. DASGUPTA
Department of Parasitology, London School of Hygiene and Tropical Medicine

(*Received* 25 *March* 1960)

INTRODUCTION

In the present paper the results of the application of the periodic acid-Schiff and the Bauer Feulgen staining methods in the different stages of the life cycles of the three species of malaria parasites (*Plasmodium gallinaceum*, *P. cynomolgi* and *Hepatocystis kochi*) are described, and compared with the results of similar studies on some other Sporozoa (*Theileria parva*, *Heptozoon sciuri*, *H. balfouri*, *Hepatozoon* sp., *Lankesterella* sp. and *Pirhaemocyton* sp.) in the available stages of their life cycles. It should be recalled in this connexion that by applying the periodic acid-Schiff technique negative result was obtained in the erythrocytic and the exo-erythrocytic stages of *Plasmodium gallinaceum* (Lillie, 1947; Lewert, 1952) and in the asexual erythrocytic stages of *P. berghei* (Sen Gupta, Ray, Dutta & Chaudhury, 1955). Bray (1957) could not demonstrate glycogen in the pre-erythrocytic schizonts of *P. cynomolgi* by using Best's Carmine and the Bauer Feulgen staining reactions; while Dasgupta (1960) found some evidence of the presence of mucopolysaccharides in the oocysts of *P. gallinaceum* and *P. cynomolgi* by applying the Alcian Blue staining method.

It will be seen from the foregoing that the detection of polysaccharides in all the different stages of the life cycles of the malaria parasites was never carried out before.

MATERIAL AND METHODS

P. gallinaceum was studied in experimentally infected mosquitoes (*Aëdes aegypti* var. *queenslandensis*) and 1-week-old chicks (*Gallus domesticus*). *Plasmodium cynomolgi* was studied in the experimentally infected mosquitoes (*Anopheles maculipennis* var. *atroparvus*) and monkeys (*Macacus rhesus*). The stages of *Hepatocystis kochi* which occur in the vertebrate host were studied in the naturally infected monkey (*Cercopithecus aethiops*). The stages of *Theileria parva*, known as Koch's blue bodies, were studied in an old collection of the sections of the spleen and the lymphatic glands of cattle. *Hepatozoon sciuri* was studied in naturally infected fleas (*Orchopeas wickhami*) and squirrels (*Sciurus carolinensis*). The stages of *Hepatozoon balfouri* which occur in the vertebrate host were studied in the naturally infected jerboas (*Jaculus jaculus*). *Hepatozoon* sp., *Lankesterella* sp., and *Pirhaemocyton* sp. were studied in the blood smears from naturally infected frogs (*Rana occipitalis*) obtained from Liberia.

* Present address: Biological Laboratory, Darjeeling Government College, Darjeeling, India.

The tissues in all cases were fixed in Carnoy's fixative and in formol saline, while the blood smears were fixed in 80% alcohol. The alcoholic periodic acid solution (Glick, 1949) was used, and the Schiff's reagent was prepared according to the method outlined by Coleman (1938). Ehrlich's haematoxylin was used as counterstain in all cases. The control slides were treated in saliva at 37°C. for 30 min. to 2 hr. in most cases. For the Bauer Feulgen reaction the same Schiff's reagent was used. The control slides were similarly treated in saliva.

OBSERVATIONS

The following observations were made.

Plasmodium gallinaceum

The wall of the oocysts took up a faint red colour when stained according to the periodic acid-Schiff method. From time to time it appeared that minute faintly stained granules occurred inside the oocysts, but the reaction was never convincing. The treatment with saliva for 2 hr. did not result in any diminution of the staining property in the wall of the oocysts. Negative results were obtained in all other stages of the life cycle, as well as in all stages treated by the Bauer Feulgen method.

Plasmodium cynomolgi

The results obtained were the same as in *Plasmodium gallinaceum*.

Hepatocystis kochi

A faint red colour was obtained in the colloidal mass of the merocysts when periodic acid-Schiff method was used. The result was otherwise negative in the stages studied.

Theileria parva

The Koch's blue bodies took up faint red colour when stained by the periodic acid-Schiff method and the Bauer Feulgen methods. Saliva controls could not be examined as the material was exhausted.

Hepatozoon sciuri (Text-fig. 1A, B)

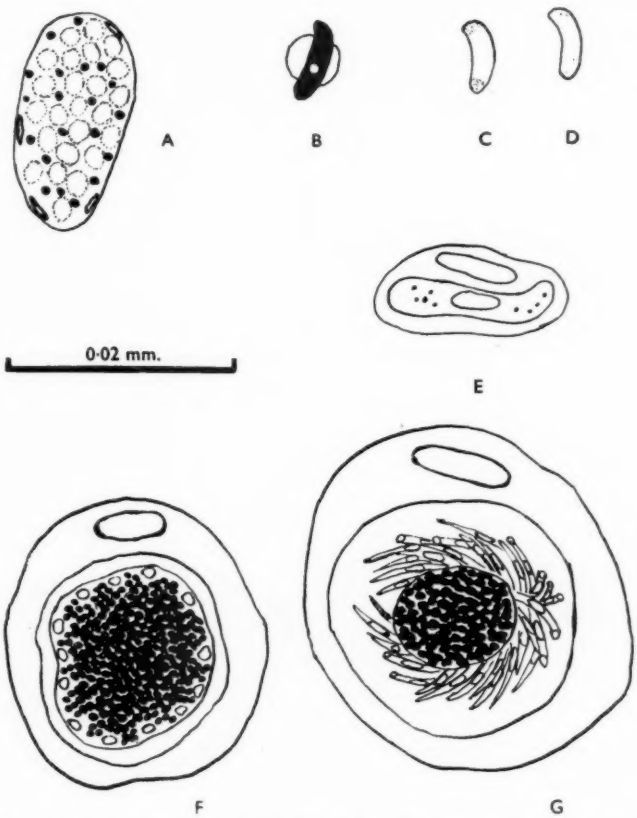
The reacting material in the form of globules was detected in the cytoplasm of the sporoblasts, when stained by the periodic acid-Schiff and the Bauer Feulgen method. The reacting material could be eliminated by the treatment with saliva. The residual mass in the oocysts stained deep red with the periodic acid-Schiff method. After treatment with saliva this reaction was found to persist. The cytoplasm of the gametocyte took up deep red colour when stained by the periodic acid-Schiff method. This reaction could not be eliminated by the treatment with saliva for two hours.

Hepatozoon balfouri (Fig. 1C, D, F, G)

The central mass of the schizonts assumed deep red colour when stained by the periodic acid-Schiff method. Under high magnification this reacting material was found to consist of small globular masses. The treatment with saliva for 30 min.

eliminated this reaction. With the Bauer Feulgen method a similar result was obtained.

The polar areas of the gametocytes stained faintly when periodic acid-Schiff method was used. This reaction could not be eliminated by treatment with saliva for 2 hr. With the Bauer Feulgen method certain minute red granules were seen in the cytoplasm; these granules could not be removed by treatment with saliva for 30 min.



Text-fig. 1. Reaction for polysaccharides in *Hepatozoon*. A. Bauer Feulgen reaction in the sporoblast of *H. sciuri*. B. Periodic acid-Schiff reaction in the gametocyte of *H. sciuri*. C. A faint reaction in the polar areas of the gametocyte of *H. balfouri*, with periodic acid-Schiff technique. D. Minute Bauer Feulgen-positive granules in the gametocyte of *H. balfouri*. E. Periodic acid-Schiff reaction in the gametocyte of *Hepatozoon* sp. F. Bauer Feulgen reaction in the schizont of *H. balfouri*. G. Periodic acid-Schiff reaction in the schizont of *H. balfouri*.

Hepatozoon sp. (Fig. 1 E)

In the cytoplasm of the gametocytes red granules were noticed when periodic acid-Schiff method was used. These granules disappeared after the treatment with saliva for 30 min.

Lankesterella sp. (Fig. 2A, B, C)

Certain areas in the cytoplasm of the sporozoites reacted positively with the periodic acid-Schiff technique and the Bauer Feulgen technique. Treatment with saliva for 2 hr. failed to remove this reacting substance.

Pirhaemocyton sp. (Fig. 3)

Among the stages of this parasite occurring in the erythrocytes two types could be distinguished with regard to their staining reaction.

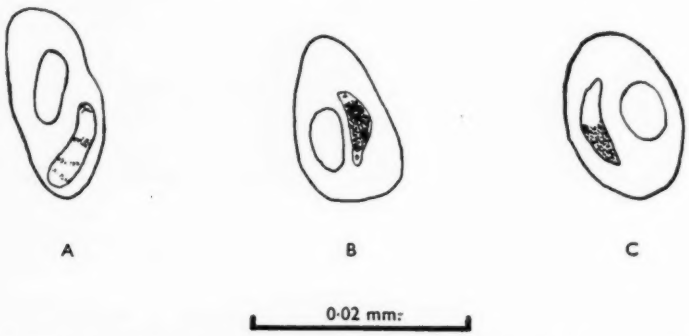


Fig. 2. The reactions for polysaccharides in the sporozoites of *Lankesterella* sp. A, B. Periodic acid-Schiff reaction. C. Bauer Feulgen reaction.

Fig. 3. Periodic acid-Schiff reaction in *Pirhaemocyton* sp.

First type. The whole structure of the parasite assumed dull red colour when stained according to the periodic acid-Schiff method. After treatment with the saliva for 2 hr. the colour either persisted or diminished to a slight extent. When Bauer Feulgen stain was used, the whole body of the parasite assumed faint red colour. After the treatment with saliva for 30 min. this reacting material completely disappeared.

Second type. With the periodic acid-Schiff technique the reacting material was found to occur in the form of minute granules in the cytoplasm. As these forms were rare, further observations could not be carried out.

DISCUSSION

A comparative study of the polysaccharide content in various species, and an evaluation of its taxonomic significance, appears to have received the attention of some workers. Dobell & Jepps (1917) pointed out that the glycogen content of the cysts of *Entamoeba* constituted a useful diagnostic character. According to Bond (1940), the spores of the species belonging to the family Myxobolidae in the order Myxosporidia, contained a large glycogen mass, whereas the spores of certain members of the family Myxosomatidae (*Myxosoma subtecalis* and *M. funduli*) and the family Myxidiidae (*Myxidium folium*) in the same order, contained glycogen in diffuse and granular forms only. An examination of the results of the present investigation is likely to prove interesting in this light.

It has been shown in the course of the present study that the *Plasmodium-Hepatocystis* group of parasites are characterized by the absence of cytochemically recognizable glycogen in them. It has also been seen that the Koch's blue bodies of *Theileria parva* reacted positively with the periodic acid-Schiff and the Bauer Feulgen methods. A survey of the literature shows that *Babesia bigemina* is the only other piroplasm in which glycogen was detected by staining methods (Ray, 1938). It would thus appear that the piroplasms differ from the *Plasmodium–Hepatocystis* group of parasites in possessing glycogen, in the cytochemically recognizable form, within the cytoplasm.

It was also noted that glycogen could be detected, in the course of this study, in the three species of *Hepatozoon*, and this result is, again, in sharp contrast with that obtained in the *Plasmodium–Hepatocystis* group. The available evidence is summarized as follows.

THE REACTION OF GLYCOGEN

Plasmodium gallinaceum	–	*Theileria parva*	+	*Hepatozoon sciuri*	+
P. cynomolgi	–	*Babesia bigemina*	+	*H. balfouri*	+
Hepatocystis kochi	–			*Hepatozoon* sp.	+

Explanations: positive reaction +; negative reaction –.

It may be pointed out in this connexion that the malarial parasites differ from *Hepatozoon* in yet another cytochemical reaction, viz. in the Feulgen's reaction for deoxyribonucleic acid, as reported in an earlier communication (Dasgupta, 1959). An explanation of these observed differences is yet to be found out.

SUMMARY

Glycogen proved to be absent in the malaria parasites (*Plasmodium* and *Hepatocystis*) by applying cytochemical methods; while in the piroplasms (*Theileria* and *Babesia*), and in *Hepatozoon* glycogen could be demonstrated by using the same methods. A strong positive reaction for polysaccharides was obtained in the residual masses of *Hepatozoon sciuri*, and *H. balfouri*; while a negative reaction was obtained in the residual mass of *Plasmodium*. A positive reaction for

polysaccharides was obtained in the following structures: the wall of the oocysts of *Plasmodium*, the gametocyte capsules of *Hepatozoon sciuri* and *Hepatozoon* sp., the sporoblast capsule of *H. sciuri*, and the polar areas of the gametocyte of *H. balfouri*.

The author wishes to thank Professor P. C. C. Garnham for his interest in this work, and for constructive suggestions. Acknowledgement is also made to the following persons, for the supply of certain Sporozoa used in this investigation: Mrs M. Vizoso, Mrs K. van der Poorten, Dr J. F. D. Frazer, Dr H. Hoogstraal and Dr R. S. Bray. A scholarship from the Government of West Bengal State (India) facilitated this study. Slides demonstrating certain results reported in this paper were exhibited in a laboratory meeting of the Royal Society of Tropical Medicine and Hygiene (Dasgupta, 1958).

REFERENCES

BOND, F. F. (1940) Glycogenous materials in myxosporidian spores. *J. Parasit.* **26**, 29.

BRAY, R. S. (1957). *Studies on the Exo-erythrocytic Cycle in the Genus* Plasmodium. London: Lewis.

COLEMAN, L. C. (1938). Preparation of lecuo basic fuchsin for use in the Feulgen reaction. *Stain Tech.* **13**, 123.

DASGUPTA, B. (1958). Certain cytochemical staining reactions in the colloid material in the merocysts of *Hepatocystis kochi* (Demonstration). *Trans. R. Soc. trop. Med. Hyg.* **52**, 296.

DASGUPTA, B. (1959). The Feulgen reaction in the different stages of the life-cycles of certain Sporozoa. *Quart. J. micr. Sci.* **100**, 241.

DASGUPTA, B. (1960). The basophilia in the different stages of the life-cycles of certain Sporozoa. *Mém. Soc. zool. tchécosl.* (In the Press).

DOBELL, C. & JEPPS, M. W. (1917). On the three common intestinal entamoebae of man, and their differential diagnosis. *Brit. med. J.* **1**, 607.

GLICK, D. (1949). *The Techniques of Histo- and Cytochemistry.* Interscience Publishers, New York.

LEWERT, R. M. (1952). Nucleic acids in plasmodia and the phosphorus partition of cells infected with *P. gallinaceum*. *J. infect. Dis.* **91**, 125.

LILLIE, R. D. (1947). Reactions of various parasitic organisms in tissues to the Bauer, Feulgen Gram and Gram-Weigert methods. *J. Lab. clin. Med.* **32**, 76.

RAY, H. N. (1938). On the nuclear structure of *Babesia bigemina* (Smith and Kilbourne). *Indian J. vet. Sci.* **8**, 183.

SEN GUPTA, P, C., RAY, H. N., DUTTA, B. N. & CHAUDHURY, R. N. (1955). A cytochemical study of *Plasmodium berghei* Vincke and Lips, 1948. *Ann. trop. Med. Parasit.* **49**, 273.

Parasitology (1960), **50**, 515–526
With 1 plate
Printed in Great Britain

Trypanosomes and dactylosomes from the blood of fresh-water fish in East Africa

By J. R. BAKER*
East African Trypanosomiasis Research Organization, Tororo, Uganda

(*Received* 26 *April* 1960)

CONTENTS

	PAGE
I. Material and methods	515
II. *Trypanosoma*	
(i) Review of previous work	516
(ii) Present work	519
(*a*) Incidence	519
(*b*) Morphology	520
(*c*) Attempt to identify vector	522
(iii) Discussion	522
III. *Dactylosoma*	
(i) Previous work	523
(ii) Present work	524
(*a*) Incidence	524
(*b*) Morphology	524
(iii) Discussion	524
IV. Summary	525
Acknowledgements	525
References	526

This paper records the incidence of two protozoan parasites, a trypanosome provisionally identified as *Trypanosoma mukasai* Hoare (1932) and *Dactylosoma mariae* Hoare (1930), in the blood of fish from Lakes Victoria and George, Uganda. A brief description of the parasites is given and their taxonomy is discussed.

I. MATERIAL AND METHODS

Fish were obtained by gill-netting from two localities: (i) Pilkington Bay, Buvuma Island, Lake Victoria, Buganda Province of Uganda, between November 1956 and March 1957; and (ii) Lake George, Toro District, Western Province of Uganda in July 1958.

Blood was collected from living fish by cutting across one or more gill-arches with scissors. Thin blood films were prepared, dried, fixed with methanol and stained for 1 hr. with Giemsa's stain. One slide from each fish was examined for at least 20 min. under a 16 mm. objective and for 10 min. under a 2 mm. oil-immersion objective unless parasites were found sooner. Impression smears were prepared from the cut surface of viscera of two fish after blotting off excess blood.

* Present address: Department of Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London, W.C. 1.

dried, fixed with methanol and stained with Giemsa's stain (as above): they were examined for 20 min. with the 2 mm. oil-immersion objective.

Endoparasites were measured after having been drawn with the aid of a camera-lucida at a magnification of $\times 2000$ or $\times 3000$: only the longitudinal axis of the body and free flagellum of some of the trypanosomes was drawn. A map-measuring wheel was used to determine the lengths of the drawings of the trypanosomes. Lengths of trypanosomes measured in the present work are given inclusive of the free flagellum unless the contrary is indicated. The anterior end of the body of trypanosomes is defined as the point at which the cytoplasm (not the flagellum) terminates, at the anterior pole of the organism.

Leeches were collected from Lake Victoria near Jinja (Eastern Province of Uganda) in January and February 1958 from fish, from weeds, or from the water. Their guts were opened and the contents examined microscopically while fresh under 16 and 4 mm. objectives.

The nomenclature of all fish listed in Table 1, and of other species where possible, has been checked from the account given by Greenwood (1958), and altered where necessary to conform with that account.

II. *TRYPANOSOMA*

(i) *Review of previous work*

Only the literature concerning trypanosomes of fresh-water fish of tropical or southern Africa is discussed below. Hoare (1955) has suggested that only constant, non-intergrading morphological or physiological differences should be used as interspecific criteria among parasitic protozoa. It is difficult to use morphological features such as size to differentiate between similar species of the large non-pathogenic trypanosomes because of the wide range of forms encountered within accepted species (see Wenyon, 1926, pp. 445–6 and 509–601). Furthermore, so little is known about the physiology of these organisms that it is impossible to use such features as host-restriction taxonomically. What evidence there is suggests that the host-restriction of the trypanosomes of fish is not narrow. Robertson (1912) cyclically transmitted trypanosomes from perch (*Perca* sp., order Percomorphi using the classification adopted by Greenwood, 1958) and bream (*Abramis* sp., order Ostariophysi) to Goldfish (*Cyprinus* sp., order Ostariophysi), which indicates that differences between hosts even of ordinal rank are inadequate interspecific criteria among these trypanosomes. All the fresh-water fish of Africa from which trypanosomes have been described are grouped within three orders of the subclass Neopterygii with the exception of *Polypterus* (subclass Palaeopterygii), in which trypanosomes were reported but not described by Neave (1906), from the Sudanese Nile. This author also described trypanosomes from '*Bageus bayard*' (= *Bagrus bayad* (Forsk.)), '*Lynodontis schal*' (= *Synodontis schall* Block-Schneider), and *Mugil* sp.

Dutton, Todd & Tobey (1906) described trypanosomes from *Clarias angolensis*, caught near Léopoldville in the Congo Free State (now the Republic of the Congo), which existed in three varieties of different size. Later, Dias (1952) named these try-

panosomes *Trypanosoma tobeyi* (see below). Wenyon (1908) reported trypanosomes from *Tilapia zilli*, the gargur fish '*Hynodontis*' (= *Synodontis schall*), *Clarias anguillaris*, *Chrysicthys auratii*, *Ophiocephalus obscurus*, *Bagrus bayad* and *Mugil* sp. in the Sudanese Nile. Rodhain (1908) described trypanosomes from *Labeo macrostoma*, *L. zalzifer* and *Malapterurus electricus* at Ubangi, Congo Free State. Those from *Labeo macrostoma* were differentiated on the grounds of size into two varieties or species.

None of the above authors gave any specific name to the parasites which they reported. However, Bouet (1909) named as *Trypanosoma toddi* a parasite from *Clarias anguillaris* in French West Africa which he thought was probably the same as that described by Dutton *et al.* (1906); these two parasites were later separated by Dias (1952) (see below). Leboeuf & Ringenbach (1910) described several species or varieties of trypanosomes from fish collected at Stanley Pool, Belgian Congo (now the Republic of the Congo). One individual of *Auchenoglanis biscutatus* was found to be scantily infected with trypanosomes which were named *Trypanosoma simondi*: the authors were unable to observe any free flagellum in these parasites, but the two published illustrations suggest that its apparent absence may have been an artifact, the region of the flagellum attached to the undulating membrane being shown as virtually unstained: thus the free portion might have been overlooked. Hence *T. simondi* is a species of doubtful validity. *Synodontis notatus* was found to be infected with what Leboeuf and Ringenbach regarded as three species of trypanosomes: they provisionally named these *Trypanosoma synodontis* A, *T. synodontis* B and *T. synodontis* C, the latter having two varieties (*parva* and *magna*). These three specific names are invalid as they are not binomina as required by the Règles Internationales de la Nomenclature Zoologique.

Leger & Leger (1914) found trypanosomes, which they did not name, in *Tilapia lata* from the river Niger in French West Africa which they believed to be identical with those described by Wenyon (1908) from *T. zilli*. Fantham (1919) reported trypanosomes from *Clarias gariepinus* at Natal, South Africa. He assigned no name to them but thought they might be *Tryanosoma barbi* Brumpt, 1906. Prates (1928) recorded unnamed trypanosomes in the blood of fish in Uganda called, in the vernacular, 'semutundu' and 'malekalende'. Greenwood (1958) identifies the former as *Bagrus docmac* but does not list the latter name.

Hoare (1932) reported finding trypanosomes in *Haplochromis nubilis*, *H. serranus*, *H. cinereus* and *H. humilior* in Lake Victoria, Uganda. As this was the first record of trypanosomes from this genus, Hoare regarded the parasite as a new species and named it *Trypanosoma mukasai*. I have measured seventeen trypanosomes on Dr Hoare's original slides, and the values of these measurements are included in Table 2. *T. mukasai* does not differ greatly from *T. toddi*, except that its free flagellum is shorter and its nucleus lies, on the average, slightly in front of the mid-point of the body (this was true of fourteen of the seventeen individuals which I measured), whereas the nucleus of *T. toddi* was, on the average, centrally placed, judging from the measurements given by Bouet (1909); unfortunately Bouet does not state how many individuals were measured. Flagellar length is too variable to be regarded as a specific character but, while it seems

unlikely that relatively small differences in the mean nuclear position will prove to be suitable for use as interspecific criteria, until this matter has been further investigated, and because of the incompleteness of the description of *T. toddi*, it seems advisable to retain *T. mukasai* as a separate species.

Dias (1952) described trypanosomes from one specimen of *Clarias gariepinus* in Moçambique and named them *Trypanosoma andrade-silvae*: Dias included in this species the trypanosomes which Fantham (1919) had described from *Clarias gariepinus*. Apart from small differences in size, the only feature separating *Trypanosoma andrade-silvae* from *T. toddi* is the fact that the free flagellum is considerably shorter in the former. Both have a central nucleus and were described from fish of the same genus. I think it likely, therefore, that *T. andrade-silvae* (including Fantham's trypanosome) is a synonym of *T. toddi*. In the same paper, Dias (1952) separated the trypanosome of Dutton *et al.* (1906) from *T. toddi*, naming the former *T. tobeyi* sp.nov., chiefly on the grounds of the difference between hosts (*Clarias angolensis* and *C. anguillaris*, respectively), the difference between the dimensions of the parasites, and the difference in the nuclear position (anterior in *Trypanosoma tobeyi*, posterior in *T. toddi*). The host difference is so slight that it cannot be regarded as justifying the creation of a new species of trypanosome. The dimensional differences between the parasites are not large, especially if it is assumed that by 'extremité antérieure' Bouet meant the anterior end of the flagellum and not, as Dias assumed, the anterior end of the cytoplasm. It seems likely that this was Bouet's meaning, as otherwise his trypanosome would have been considerably longer than that of Dutton *et al.* (1906) and so he would have been unlikely to have regarded them both as members of one species. If this assumption as to Bouet's meaning is made, the position of the nucleus in *T. toddi* (as calculated from the measurements) becomes approximately central and not posterior. The small difference in nuclear position then remains the only feature separating *T. tobeyi* from *T. toddi*, and because of it the separation proposed by Dias (1952) may be valid. It is quite possible that the forms described by Dutton *et al.* (1906) belong to the species which Hoare (1932) later named *T. mukasai*, in which case *T. tobeyi* would become a synonym of the former. However, it is inadvisable with the rather scanty information available at present definitely to assert this synonymity.

Later, Dias (1955) described what he believed to be three species of trypanosomes from *Tilapia mossambica* in Moçambique: these he named *Trypanosoma napolesi*, *T. rebeloi* (in which were included the trypanosomes described by Wenyon, 1908, in *Tilapia zilli*) and *T. serranoi* (in which were included the trypanosomes which Neave, 1906, reported from *Synodontis schall*). Of five *Tilapia mossambica* examined, trypanosomes were found in the blood of four: and of these four, Dias (personal communication) stated that in two, only *T. rebeloi* was found; in one, both *T. rebeloi* and *T. serranoi*; and in one, *T. napolesi* and *T. serranoi*. *T. rebeloi* and *T. serranoi* were separated from each other chiefly on the grounds of difference in size, but as the two forms are very similar in general appearance it seems likely that they represent only the two ends of a continuous series. *T. napolesi* was separated from the two other species chiefly because of its greater width and its

apparent lack of free flagellum. As only one individual was observed, the possibility that these features were artifacts cannot be ruled out. It seems likely, therefore, that the three 'species' described by Dias (1955) are variants of a single species. There is no constant morphological difference between this species (assuming the absence of a free flagellum from *T. napolesi* to be an artifact) and *T. mukasai*, and it is probable that Dias's three specific names should be regarded as synonyms of *T. mukasai* Hoare (1932).

(ii) *Present work*

(*a*) *Incidence*

The incidence of the trypanosomes is shown in Table 1. About 52 % of the two species of *Tilapia* examined from Lake Victoria, and 20 % of the fish of the same genus examined from Lake George, were found to be infected with trypanosomes. The number of individuals examined from Lake George (10) is too small for these figures to be taken as evidence that there is a lower infection rate in that lake.

Table 1. *Incidence of* Trypanosoma mukasai *and* Dactylosoma mariae

Species of fish	Lake	No. examined	No. infected with		
			T. mukasai	*D. mariae*	Both
Tilapia nilotica (L., 1757)*	George	10	2 (L)†	6	0
T. esculenta Graham, 1928	Victoria	24	12 (B)	11	5
T. variabilis Boulenger, 1906	Victoria	24	13 (B)	17	9
Labeo victorianus Boulenger, 1901	Victoria	3	0	1	0
Bagrus docmac (Forsk., 1775)	Victoria	4	4 (B)	0	0
Mormyrus kannume Forsk., 1775	Victoria	2	2 (L)	0	0
Gnathonemus victoriae Worthington, 1929	Victoria	1	0	0	0
Haplochromis Hilgendorf, 1888, sp. indet.	Victoria	1	1 (S)	0	0
Schilbe mystus (L., 1762)	Victoria	1	0	0	0
Astatoreochromis alluaudi Pellegrin, 1904	Victoria	1	1 (L)	1	1
Protopterus aethiopicus Heckel, 1851	Victoria	1	0	0	0
Totals	George	10	2	6	0
	Victoria	62	33	30	15
	Both lakes	72	35	36	15

Notes: * A few *Tilapia leucosticta* Trewavas, 1933, may have been included. † (L) = large form, (S) = small form, (B) = both forms of *T. mukasai* found.

The infection rate in all fish examined from both lakes was about 49 %. As the parasites were very scanty in these fish, it is likely that the true infection rates were higher than these figures indicate.

(b) Morphology

The lengths of the trypanosomes seen ranged from 22 to 65μ. Although the distribution was almost continuous within this range, with no discontinuities between individual lengths greater than 4μ, a frequency diagram of the lengths of the trypanosomes plotted against the numbers of individuals was bimodal (Text-fig. 1). On this basis, the trypanosomes are considered to exist in two forms, small (below 45μ long) and large (length 45μ or more). Both these forms are provisionally regarded as belonging to the same species (see Discussion below). Of the thirty-five fish found to be infected, both forms of trypanosome were found in four: the large form alone was seen in twenty-two fish and the small form alone in nine. The descriptions given below apply to trypanosomes from all host species and both localities. No significant difference was observed between parasites from any of the infected fish.

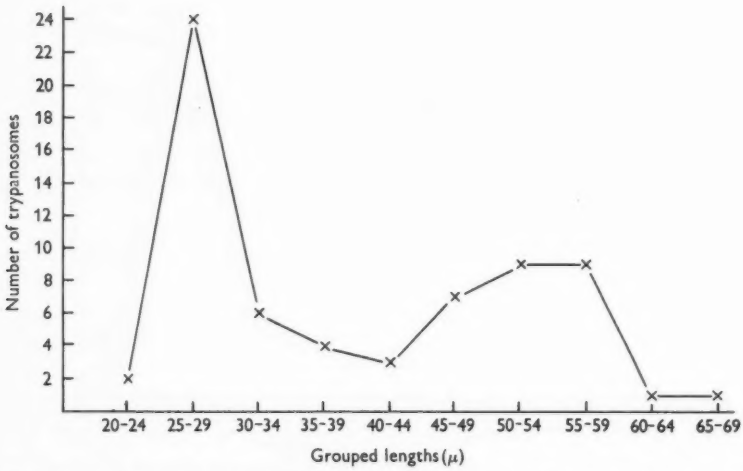


Fig. 1. Graph showing frequency of occurrence of different body lengths (including free flagellum) of *Trypanosoma mukasai*.

Small form (Text-fig. 2D, E). Seen on dried, stained thin blood films, the trypanosomes had a large round or oval kinetoplast* lying very near, or actually at, the bluntly pointed posterior end. The flagellum usually appeared to arise laterally from the kinetoplast and then passed forward along a fairly well-developed undulating membrane to the tapered anterior end of the body. A free flagellum was always present. The nucleus was usually oval, with its longest diameter parallel to the longitudinal axis of the trypanosome. In the majority of the individuals (twenty-five of the twenty-seven measured) the nucleus lay just forward of the mid-point of the body: in a few it was slightly behind the mid-point. The average measurements of twenty-seven small trypanosomes are shown in Table 2.

* Throughout this paper the term 'kinetoplast' is used to describe what was presumably the kinetoplast–blepharoplast complex.

In addition, the lengths only of twelve small trypanosomes were measured, and the mean length of all these thirty-nine small trypanosomes was 29·5μ, with a range of 22–42μ. The modal value, as can be seen from Text-fig. 1, lay in the range 25–29μ.

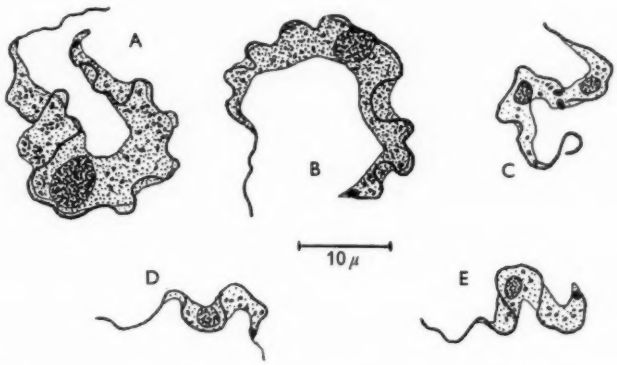


Fig. 2. Camera-lucida drawings of *Trypanosoma mukasai* on dried, Giemsa-stained thin blood films (× 1500). A, C–E from *Tilapia variabilis*, B from *T. esculenta*. A and B, large forms; C–E, small forms (those in C apparently undergoing division or agglutination).

Table 2. *Average measurements in microns of* Trypanosoma mukasai

	Present material					
	Small form (27 measured)		Large form (13 measured)		Dr Hoare's material (17 measured)	
	Mean	Range	Mean	Range	Mean	Range
Posterior end of body to posterior edge of kinetoplast	0·5	0–2	1·0	0·5–2	1·1*	0–2·4*
Diameter of kinetoplast (longit.)	0·7	0·25–1·5	0·9	0·5–1·5	0·9	0·5–1·2
Diameter of kinetoplast (trans.)	0·5	0·25–1	0·8	0·5–1	—	—
Anterior edge of kinetoplast to posterior edge of nucleus	9·8	5–17	20·1†	14–29†	18·7	11·4–21·6
Diameter of nucleus (longit.)	2·7	1·75–3·5	4·0†	3–5·5†	—	—
Diameter of nucleus (trans.)	1·2	0·75–1·75	3·2†	1·5–5·75†	—	—
Anterior edge of nucleus to anterior end of body	6·6	4·5–11·5	16·6†	13–19†	16·6	9–20·1
Length of free flagellum	7·7	5–12	10·5	7–17	4·0‡	0·9–12‡
Total length (excluding free flagellum)	19·4	16–27	41·8	33–54	40·9	35·7–59·4
Total length (including free flagellum)	28·2	22–35	52·4	45–62	—	—
Max. width of undulating membrane	0·6‡	0·5–0·75‡	1·0§	0·5–1·75§	—	—
Width at level of nucleus (excluding undulating membrane)	1·6	1–2·75	3·8†	2–6†	2·5	1·8–4·2

* Measured in five individuals only.
† Measured in twelve individuals only.
‡ Measured in eight individuals only.
§ Measured in ten individuals only.

Two small trypanosomes were seen, which may represent division or agglutination of the parasites (Text-fig. 2C). These have been excluded from the measurements given above and in Table 2 as no boundary between the two could be discerned: however, the smaller was about 15μ long and the larger, about 24μ.

Large forms (Text-fig. 2A, B). On dried, stained thin blood films these trypanosomes were seen to have a large round or oval kinetoplast near the obtuse or bluntly pointed posterior end, from which the flagellum usually appeared to arise laterally. The undulating membrane was well developed, and clearly visible for most of its length as a feebly stained area between the flagellum and the intensely blue-stained cytoplasm: a free flagellum was always present. The nucleus was round or oval and lay somewhat anterior to the mid-point of the body in all of the twelve individuals in which this feature was measured. Oval nuclei were usually but not always orientated with their long axes parallel to the longitudinal axis of the trypanosome.

The average measurements of thirteen large trypanosomes are shown in Table 2. The lengths only of an additional fourteen large individuals were measured, the average length of all twenty-seven large trypanosomes being 52·6μ (range 45–65μ, mode 50–59μ). The distribution of lengths is shown in Text-fig. 1.

(c) *Attempt to identify the vector*

Fourteen leeches (Annelida, Class Hirudinea), the only known vectors of trypanosomes of fish (see Wenyon, 1926, pp. 602–6), were examined, of which six had died before dissection. Of these fourteen, only two had been collected from fish—one from a *Protopterus aethiopicus* and one from a *Barbus altianalis radcliffi* Boulenger: it is not known whether either of these fish was infected with trypanosomes. The only leech in which flagellates were seen was that collected from the *B. a. radcliffi* (on 4 February 1958), in which many crithidia, some of which were dividing, were seen. A similar leech collected at the same time from the same fish was identified by Professor M. C. Meyer, University of Maine, U.S.A., as a member of the family Glossiphoniidae. Certain specific identification was impossible because the specimen was engorged and slightly damaged, but it was a member of the genera *Placobdella* or *Batracobdella*, more probably the latter: it may have been *B. tricarinata* (Blanchard, 1897).

(iii) *Discussion*

It can be seen from Text-fig. 2 and the description given above that, apart from their size, the morphology of both the large and the small forms of the trypanosomes which form the subject of the present paper is very similar: the free flagellum is, however, proportionately longer in the small forms. The undulating membrane appears to be better developed and more clearly marked off from the rest of the body in the larger forms, but this appearance may be due to the greater thickness of the body of the larger trypanosomes. Because of this similarity, because the greatest discontinuity in the length distribution of all the trypanosomes was only 4μ, and because no significant morphological difference was seen between

the parasites from the different species of fish, both forms of trypanosomes are regarded as representing different stages of growth or differentiation in the life cycle of one species.

The large forms of the trypanosome described above closely resemble those which Hoare (1932) named *Trypanosoma mukasai* (see Table 2). The only morphological difference between the two which is more than trivial is the fact that most of the individuals of Dr Hoare's material of *T. mukasai* which I examined had shorter free flagella. However, I regard flagellar length as too variable a character among these large trypanosomes to be used in separating species. As already stated, I regard the large and small forms of the trypanosomes described above as different forms of one species. The trypanosomes described by Dias (1955) from *Tilapia mossambica*, which also closely resemble my own material, I consider to be *Trypanosoma mukasai*. Consequently the trypanosomes reported in the present paper from *Tilapia* spp. from Lakes George and Victoria, and *Bagrus* sp., *Mormyrus* sp., *Haplochromis* sp. and *Astatoreochromis* sp. of Lake Victoria, are identified as *Trypanosoma mukasai* Hoare, 1932. All the hosts listed in Table 1 except *Haplochromis* sp. indet. are new host records for this species, and Lake George, Uganda, is a new locality record.

Shortage of time precluded the completion of the attempt to identify the vector of *T. mukasai*. The crithidia which were found in the gut of the glossosiphoniid leech referred to above may have been derived from the fish from which it was collected or from another fish, or possibly from an amphibian or reptile. Even if it is assumed that the flagellates were of piscine origin, their multiplication in the gut of the leech does not necessarily prove that that species of leech is a vector of the flagellates, as no metacyclic trypanosomes were seen. Further investigation is needed with leeches of this and other families.

III. *DACTYLOSOMA*

(i) *Previous work*

The first species of *Dactylosoma* to be reported from fish was described by Hoare (1930) in five of fourteen individuals of *Haplochromis* spp. of Lake Victoria, Uganda, and named *Dactylosoma mariae*. The parasite was exclusively intraerythrocytic and underwent schizogony resulting in the production of four merozoites arranged in the form of a rosette. The only other species of this genus to be recorded from fish is *D. salvelini* Fantham, Porter & Richardson (1942) of the Canadian trout (*Salvelinus fontinalis*). This species produced eight merozoites and was slightly smaller than *Dactylosoma mariae*.

Jakowska & Nigrelli (1956) transferred *D. mariae* and one other species from *Triturus* to a new genus (*Babesiosoma*) 'on the basis of general morphology, staining reaction, [and] constancy in the number and configuration of merozoites'. *Babesiosoma* differs from *Dactylosoma* in lacking a nuclear karyosome, possessing vacuolated and only slightly granular cytoplasm, and producing only four merozoites in a rosette or cross.

(ii) *Present work*

(*a*) *Incidence*

The incidence of this parasite is shown in Table 1: 60% of the *Tilapia* examined from Lake George and about 58% of those examined from Lake Victoria were found to be infected. The incidence in all the fish examined from both lakes was 50%. As the parasites were often scanty in the peripheral blood, the true infection rate may have been higher than this.

(*b*) *Morphology*

The organisms were seen only within erythrocytes. Their morphology in dried, stained thin blood films was very similar to that described by Hoare (1930). The schizonts, merozoites and young trophozoites were characterized by the presence of a central vacuole surrounded by a thin layer of cytoplasm (often scarcely noticeable), with a slightly thicker 'cap' of nuclear material at one end (Pl. 1, fig. 1–3). The dimensions of one merozoite were about $2{\cdot}5 \times 1{\cdot}2\mu$, and those of the two single forms shown in Pl. 1, fig. 2 were $2{\cdot}5 \times 1{\cdot}0\mu$ and $2{\cdot}5 \times 1{\cdot}3\mu$. Schizogony appeared to begin by the division of the nucleus into four, followed by longitudinal cytoplasmic cleavage into two binucleate elongated bodies. The latter then each divided transversely into two merozoites, the four merozoites so formed arranging themselves in the characteristic rosette or cross. This corresponds to the second type of schizogony described by Jakowska & Nigrelli (1956). The schizont shown in Pl. 1, fig. 1 measured 5μ along its two longer diameters, the merozoites being $1{\cdot}5$–$2{\cdot}0\mu$ broad. Pl. 1, fig. 3 shows a trophozoite measuring $2{\cdot}5 \times 2{\cdot}0\mu$: the largest of these forms which was measured was $5{\cdot}0 \times 2{\cdot}5\mu$ in size.

Forms interpreted as gametocytes were larger and lacked the central vacuole. Pl. 1, fig. 4, shows what is believed to be a macrogametocyte ($6{\cdot}5 \times 4{\cdot}5\mu$) and Pl. 1, fig. 5 shows what is presumably a microgametocyte ($6{\cdot}5 \times 2{\cdot}0\mu$). These macro- and microgametocytes were differentiated by the characters used by Hoare (1930), namely, that the female forms are more rounded and the males club-shaped or crescentic. The parasitized erythrocytes are apparently unaltered, except for the fact that the nucleus was laterally displaced in some of the cells containing gametocytes. All the infections seen were sparse.

Impression smears of spleen, liver and kidney from one *T. variabilis*, and of these organs and heart muscle from another *T. variabilis* (both fish were infected with *T. mukasai* and the species of *Dactylosoma* and were caught in Pilkington Bay) were examined for developmental stages of this parasite, without success. Spores of a myxosporidian were, however, found in both fish and will be described in a separate paper.

(iii) *Discussion*

The morphology and dimensions of this parasite closely resemble those described by Hoare (1930) for *D. mariae*. The parasite described by Fantham *et al.* (1942) from Canadian fish is clearly distinct as it produces eight merozoites.

Jakowska & Nigrelli's (1956) separation of *D. mariae* into a new genus has not been adopted in this paper, because the differential characters which they propose

are perhaps insufficient to warrant generic separation, although it is clear from their work that there are two groups of species within the genus *Dactylosoma* which merit separation at least at the subgeneric level.

The parasite described in this section of the paper is therefore identified as *D. mariae* Hoare, 1930. If the new genus *Babesiosoma* Jakowska & Nigrelli (1956) is accepted, the parasite should be transferred to that genus. To the habitat given by Hoare (1930) can be added the erythrocytes of *Tilapia esculenta*, *T. variabilis*, *T. nilotica*, *Labeo victorianus* and *Astatoreochromis alluaudi*. Lake George in the Toro District of the Western Province of Uganda, and the region of Lake Victoria north of Buvuma Island, Buganda Province of Uganda, can be added to Hoare's (1930) locality record.

IV. SUMMARY

1. Trypanosomes were found in the blood of thirty-five of seventy-two fish of five genera (chiefly *Tilapia* spp.) from Lakes Victoria and George, Uganda.

2. The trypanosomes, which were never numerous in the blood, showed two morphological forms—small (22–44μ long) and large (45–65μ). No significant differences were observed between the trypanosomes from the different species of fish, and all the trypanosomes seen are provisionally identified as *Trypanosoma mukasai* Hoare, 1932.

3. Crithidia were found in the gut of a glossosiphoniid leech which had been removed from a fish; although suggestive, this cannot be taken as conclusive evidence that leeches of this family are vectors of *T. mukasai*.

4. The taxonomy of the trypanosomes of African fresh-water fish is reviewed, and it is concluded that only the following species should be regarded as valid at present: *T. toddi* Bouet, 1909, *T. mukasai* Hoare, 1932, and *T. tobeyi* Dias, 1952.

5. *Dactylosoma mariae* Hoare, 1930, was found in the blood of thirty-six of the seventy-two fish examined. Merozoites, schizonts and gametocytes of this small unpigmented parasite were observed within erythrocytes. No developmental stages were found in certain viscera of two infected fish.

I am very grateful to the staff of the East African Fisheries Research Organization for their help and interest, especially Mr R. S. A. Beauchamp (Director), Dr D. G. Fryer, Dr P. H. Greenwood (now at the British Museum, Natural History, who also helped me with the nomenclature of the fish) and Mr D. Roberts. My thanks are also due to Dr C. A. Hoare and Dr J. A. T. S. Dias for sending me some of their type material; to Professor M. C. Meyer for identifying the leech; to Mr D. H. Bowser and the staff of the Uganda Fish Marketing Corporation at Kasenyi, Lake George; and to Mr G. D. Lomax and Mr C. J. Webb of the East African Trypanosomiasis Research Organization for technical assistance; Mr Webb also took the photomicrographs.

REFERENCES

BOUET, G. (1909). Sur quelques trypanosomes des vertébrés à sang froid de l'Afrique occidentale française. *C.R. Soc. Biol., Paris*, **66**, 609.

DIAS, J. A. T. S. (1952). Uma nova espécie di tripanosoma, parasita do *Clarias gariepinus* (Burchell 1822), peixe africano de água doce. *An. Inst. Med. trop., Lisboa*, **9**, 167.

DIAS, J. A. T. S. (1955). Estudos sobre os hematozarios dos peixes de água doce de Moçambique. I. Descrição de algumas novas espécies de tripanossomas, parasitas da 'Tilapia mossambica' (Peters, 1852). *Moçambique: Doc. trimestr.*, no. 82, 47.

DUTTON, J. E., TODD, J. L. & TOBEY, E. N. (1906). Concerning haemoflagellates of an African fish (*Clarias angolensis*). *J. Med. Res.* **15**, 491.

FANTHAM, H. B. (1919). Some parasitic protozoa found in South Africa. II. *S. Afric. J. Sci.* **16**, 185.

FANTHAM, H. B., PORTER, ANNIE & RICHARDSON, L. R. (1942). Some haematozoa observed in vertebrates in eastern Canada. *Parasitology*, **34**, 199.

GREENWOOD, P. H. (1958). *The Fishes of Uganda*. Kampala: The Uganda Society.

HOARE, C. A. (1930). On a new *Dactylosoma* occurring in fish of Victoria Nyanza. *Ann. Trop. Med. Parasit.* **24**, 241.

HOARE, C. A. (1932). On protozoal blood parasites collected in Uganda. *Parasitology*, **24**, 210.

HOARE, C. A. (1955). Intraspecific biological groups in pathogenic Protozoa. *Refuah Vet.* **12**, 263.

JAKOWSKA, S. & NIGRELLI, R. S. (1956). *Babesiosoma* gen. nov. and other babesioids in erythrocytes of cold-blooded vertebrates. *Ann. N.Y. Acad. Sci.* **64**, 112.

LEBOEUF, A. & RINGENBACH, —. (1910). Sur quelques hématozoaires du Congo. *Ann. Inst. Pasteur*, **24**, 945.

LEGER, M. & LEGER, A. (1914). Hémogrégarine et trypanosome d'un poisson du Niger, *Tilapia lata*. *C.R. Soc. Biol., Paris*, **77**, 183.

NEAVE, S. (1906). Report of travelling pathologist and naturalist. *Second Rep. Wellcome Res. Labs., Khartoum*, pp. 183–204.

PRATES, M. M. (1928). The precipitin test applied to the blood in the alimentary canal of Glossinae. *Final Rep. League of Nations international Commission on Human Trypanosomiasis*, p. 179. Geneva.

ROBERTSON, MURIEL (1912). Transmission of flagellates living in the blood of certain fresh water fishes. *Philos. Trans.* B, **202**, 29.

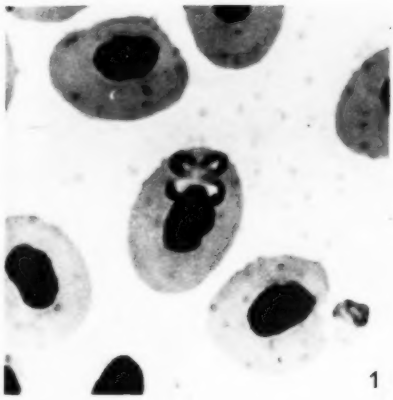
RODHAIN, J. (1908). Note sur quelques trypanosomes de grenouilles et de poissons dans l'Ubangi. *Zbl. Bakt.* (Abt. 1, Orig.) **45**, 129.

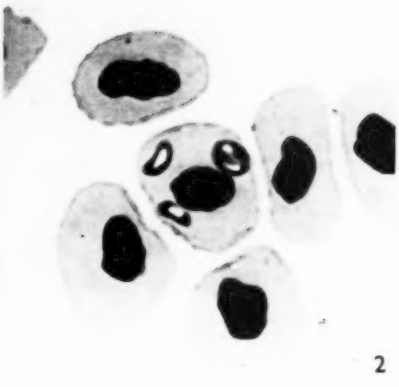
WENYON, C. M. (1908). Report of travelling pathologist and protozoologist. *Third Rep. Wellcome Res. Labs., Khartoum*, pp. 121–168.

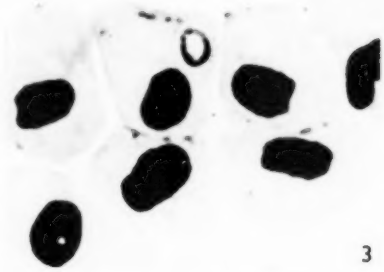
WENYON, C. M. (1926). *Protozoology*, vol. 1. London: Baillière, Tindall and Cox.

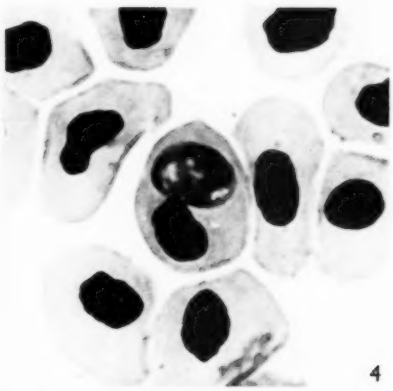
EXPLANATION OF PLATE 1

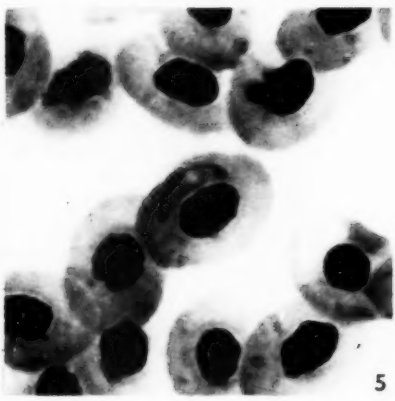
Photomicrographs of *Dactylosoma mariae* on dried, Giemsa-stained thin blood films. × 2000. Fig. 1, schizont; Fig. 2, merozoites; Fig. 3, trophozoite; and Fig. 4, macrogametocyte (all from *Tilapia variabilis*); Fig. 5, microgametocyte (from *T. esculenta*).


1


2


3


4


5

Parasitology (1960), **50**, 527–530
With 7 text-figures
Printed in Great Britain

Notes on *Limnophora exsurda* Pand. (Diptera, Anthomyidae)

By P. TATE
The Molteno Institute, University of Cambridge

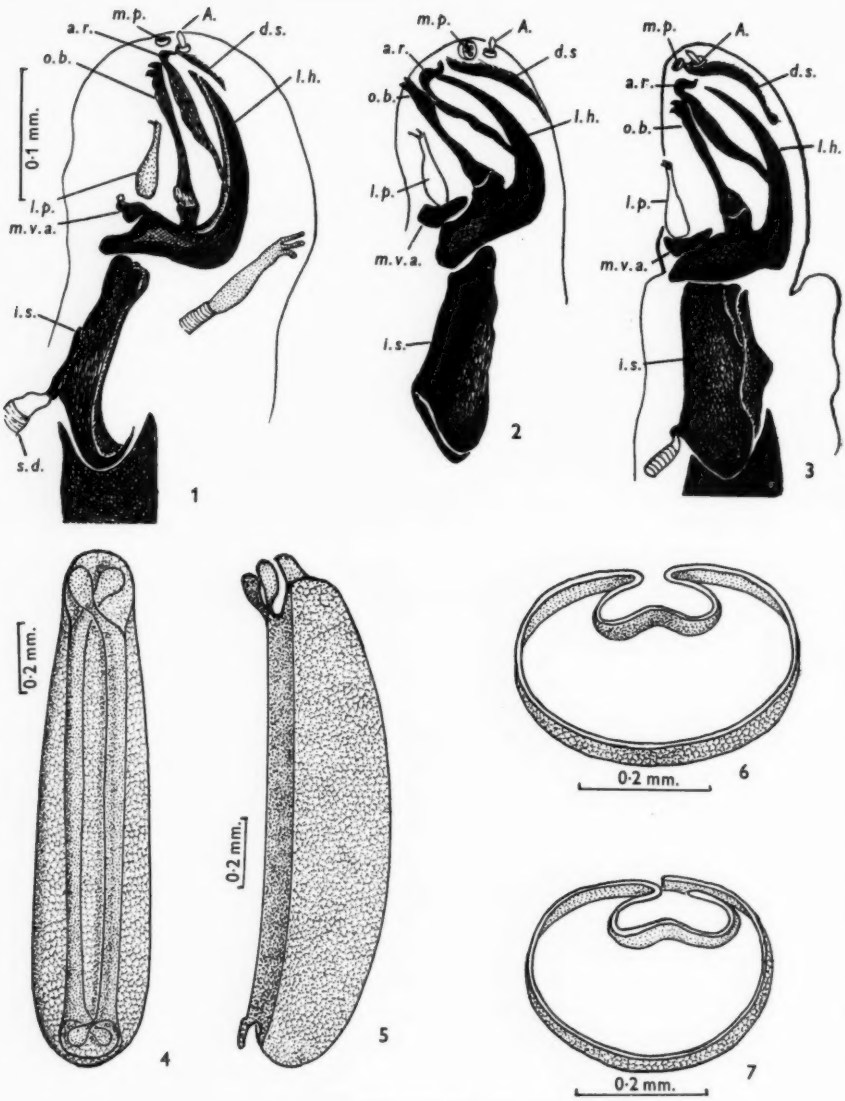
(*Received* 24 *February* 1960)

The 3rd larval and the pupal stages of *Limnophora exsurda* Pand. (Collin, 1921) have been described previously (Tate, 1939), and it was suggested that larval trimorphism is absent in this species as it is in some other carnivorous anthomyid larvae. The eggs and newly hatched larvae were not known at that time. Since then more material has been collected and it is now possible to describe the eggs and the very early larva. The material, comprising puparia, one large larva and eggs, was found in masses of algae in rock pools above high water mark on the south coast of Co. Cork, Ireland. Superficially the puparia and larva appeared to be the same as those of *L. exsurda* and it was suspected that the eggs were those of the same species. Flies which emerged from some of the puparia proved to be *L. exsurda*. Although all except one of the eggs were empty, a dead larva was found in this egg and it was possible to extract it from the egg and compare its structure with that of known larvae of *L. exsurda* and with the larval characters revealed by the exuviae extracted from puparia after the flies had emerged.

The eggs are 2·4 mm. long, shining white with very fine hexagonal surface markings (Figs. 4, 5). They have rounded ends and taper slightly towards one end. In side view they are nearly flat on one surface and convex on the other. The lateral edges of the flat surface form inwardly directed flanges which are produced at each end into spatulate lappets (Fig. 4). In cross-section it is seen that the flat surface is formed by an infolding of the wall, bordered by the lateral flanges (Figs. 6, 7). When eclosion occurs the cleavage is along the inner margins of these flanges (Fig. 7). With the material available it was not possible to determine the orientation of the larva within the egg and it is not known which is the dorsal or ventral surface, or which is the anterior end. Thomson (1937) has pointed out that in *Phaonia variegata*, in which the egg has lateral flanges, the flat surface is ventral, whereas in the *Myiospila* type of egg the flat, flanged surface is dorsal.

The larva extracted from the egg already shows features associated with a carnivorous mode of life. Such features are shown in the bucco-pharyngeal armature (Fig. 1) in the slender, sharply pointed lateral hooks (*l.h.*), the well-developed anterior ribbon (*a.r.*), the strong oral bars with terminal teeth (*o.b.*), the complete median ventral arc (*m.v.a.*), the strong intermediate sclerite (*i.s.*) and the absence of ventral pharyngeal ridges. The dorsal sclerite (*d.s.*) is not yet strongly sclerotized but the antennae (*A.*), maxillary palps (*m.p.*) and labial palps (*l.p.*) are as well developed as in the late 3rd-stage larva.

Comparison of the anterior regions of the larva extracted from the egg (Fig. 1), of the 3rd-stage larval moult extracted from a puparium (Fig. 2), and of a 3rd-stage



Limnophora exsurda Figs. 1–7.

Fig. 1. Larvae extracted from egg—anterior region.

Fig. 2. Third larval exuvium extracted from puparium, anterior region. Same lot of material and scale as Fig. 1.

Fig. 3. Third-stage larva from material previously described. Anterior region. Same scale as Fig. 1.

Fig. 4. Egg, empty. ? dorsal.

Fig. 5. Egg, empty. Lateral.

Fig. 6. Egg, transverse section showing infolded lateral flanges.

Fig. 7. Egg, transverse section, showing cleavage at margin of flange.

larva from the material previously described (Fig. 3). shows that, except for differences in the degree of sclerotization, there is very close agreement in structure and dimensions in all cases. It is clear that the larvae all belong to the same species and that the larva has already attained the characteristics of the 3rd-stage larva within the egg. It is remarkable, therefore, that during larval life although there is a great increase of body size, there is no change in structure or size of the organs such as spiracles and the bucco-pharyngeal armature. From the moment it emerges from the egg the larva is fully equipped for its carnivorous mode of life and this uniformity in behaviour has allowed the 1st and 2nd larval stages to be omitted from the life cycle. Thus in, *Limnophora exsurda*, larval trimorphism is absent and stages one and two are missing or occur in an abortive form within the egg, although no traces of moults representing such stages could be found in the empty eggs.

The absence of larval trimorphism appears to be a character associated with the carnivorous mode of life of anthomyid larvae and has been described by Portchinsky (1910) in *Mydaea urbana* and *Myiospila meditabunda* in which he considered that the 2nd stage was omitted in the life cycle but Thomson (1937) stated that the larva hatches in the 2nd stage and that it is the 1st stage that is omitted; by Keilin (1917) in *Melanochelia riparia*, in which both 1st and 2nd stages are absent; by Tate (1935) in *Phaonia mirabilis* in which the newly hatched larva is in the 3rd stage, and by Thomson (1937) in *Myiospila meditabunda* and *Hebecnema umbratica* which hatch as 2nd-stage larvae, and in *Mydaea pagana* and *Phaonia variegata*, which hatch in the 3rd stage but in which vestiges of 2nd-stage exuviae could be detected within the eggs after eclosion. In the latter two species the hatching of larvae in the 3rd stage is correlated with a carnivorous mode of life.

The flies of *Limnophora exsurda* were very kindly identified by Mr J. E. Collin, who supplied the following notes on the species (Collin, 1954) which I quote, with his permission, as follows:

> There has always been some question about the use of Pandellé's name *exsurda* for this species, because he described the base of the cubital vein (vein R_{4+5} in some modern terminologies) as 'bare'. Stein's *L. exsurda* Pand. has been sunk as a synonym of *L. setinerva* S. & D. but the figures of male genitalia given by these authors for their *setinerva* and *exsurda* prove that our British *exsurda* cannot be their *setinerva*, but might be their *exsurda*. Unfortunately, according to Séguy (1923), the type of Pandellé's species cannot be found, so that the doubt concerning its identity must remain.
>
> Under the circumstances you may like to refer to a hitherto unrecorded character separating our British *exsurda* from other species of this group, viz. the presence of hairs on the sides of scutellum below the strong lateral bristles.

In a recent personal communication Collin (1960) has supplied additional notes on the confusion in the literature between *L. exsurda* and *L. setinerva*. He writes as follows:

> Schnabl described his *setinerva* in 1911 from one pair only, taken by Villeneuve near *Grenoble*, and the figures he gave of its male genitalia were sufficiently different from those he gave for *exsurda* to cause him to propose a separate subgenus for its reception (Schnabl & Dziedzicki, 1911).
>
> At the same time Schnabl described under the name of *latevittata* a variety of *exsurda* from one male taken at Dauphiné (Lautaret), with male genitalia as shown by figures only slightly different from those of *exsurda*.
>
> Thus is was evident that Schnabl accepted *exsurda* (with var. *latevittata*) as quite distinct from his *setinerva*, and Stein was evidently of the same opinion in 1916, but Séguy in 1923,

after stating that no type of *exsurda* could be found in Pandellé's collection, and that Pandellé's description of a *bare* base to the cubital vein in that species was probably an error, used the name *setinerva* Strobl for Stein's *exsurda* Pand., apparently because he had received from Villeneuve a statement on Stein's identification of *exsurda* Pand. which he quoted as follows: 'l'interprétation de Stein n'est pas certaine. *L. exsurda* (Pandellé) Stein serait le même que le *L. setinerva* Schnabl decrit du Dauphiné'. But the species Schnabl described 'from Dauphiné' was *not setinerva* but *exsurda* var. *latevittata*, thus *setinerva* was still left as a distinct species on the strength of genital differences.

It is unfortunate that Hennig (1959) in Lindner's 'Die Fliegen, Muscidae' has also failed to notice Villeneuve's misquotation of *setinerva* for *exsurda* var. *latevittata*, and has accepted Séguy's incorrect conclusions.

Under the circumstances the use of the name *setinerva* Schnabl for our British species would be incorrect.

Thus the correct designation of the species collected in the south of Ireland is *Limnophora exsurda* Pand.

SUMMARY

The eggs of *Limnophora exsurda* are described. The larva is in the 3rd stage when it emerges from the egg. The absence of larval trimorphism has been described in a number of dipterous larvae and appears to be correlated with a carnivorous mode of life in the larval stage. Notes on the nomenclature of *L. exsurda* by Mr J. E. Collin are given.

I wish to express my thanks to Mr J. E. Collin for his kindness in identifying the flies and supplying the notes on the nomenclature of the species.

REFERENCES

COLLIN, J. E. (1921). The British species of the anthomyid genus *Limnophora* Desv. *Ent. Mon. Mag.* 3rd ser. **7**, 94–100, 162–75, 238–48, 265–8.

COLLIN, J. E. (1954; 1960). Personal communications.

HENNIG, W. (1959). In: Lindner, E. *Die Fliegen der Palaearktischen Region*. 63*b Muscidae*. Part **207**, 337–84.

KEILIN, D. (1917). Recherches sur les anthomyides à larves carnivores *Parasitology*, **9**, 325–450.

PORTCHINSKY, I. A. (1910). Recherches biologiques sur le *Stomoxys calcitrans* L. et biologie comparée des mouches coprophagues. *Trud. Byu. Ent., Spb.* **8**, 1–6; with Appendix 1–90 (in Russian).

SCHNABL, J. & DZIEDZICKI, H. (1911). Die Anthomyiden. *Nova Acta Leop. Carol.* **95**, 55–358.

SÉGUY, E. (1923). *Diptères anthomyides. Faune de France*. Paris. Paul Lechevalier.

STEIN, P. (1916). Die Anthomyiden Europas. Tabellen zur Bestimmung der Gattungen und aller mir bekannten Arten, nebst mehr oder weniger ausführlichen Beschreibungen. *Arch. Naturgesch.* (1915). Abt. A. 10 Heft. 1–224.

TATE, P. (1935). The larva of *Phaonia mirabilis* Ringdahl, predatory on mosquito larvae (Diptera, Anthomyidae). *Parasitology*, **27**, 556–60.

TATE, P. (1939). The early stages of *Limnophora exsurda* Pand. (Diptera, Anthomyidae). *Parasitology*, **31**, 479–85.

THOMSON, R. C. MUIRHEAD (1937). Observations on the biology and larvae of the Anthomyidae. *Parasitology*, **29**, 273–358.

KEY TO LETTERING OF FIGURES

A.	antenna	*l.p.*	labial palp
a.r.	anterior ribbon	*m.p.*	maxillary palp
d.s.	dorsal sclerite	*m.v.a.*	median ventral arc
i.s.	intermediate sclerite	*o.b.*	oral bar
l.h.	lateral hooks	*s.d.*	salivary duct

Parasitology (1960), **50**, 531–550
With 10 text-figures
Printed in Great Britain

Enzymic proteolysis by *Entamoeba histolytica*; biochemical characteristics and relationship with invasiveness

By R. A. NEAL
Wellcome Laboratories of Tropical Medicine, London, N.W. 1

(*Received* 25 *February* 1960)

I. INTRODUCTION

The mechanism by which *Entamoeba histolytica* is able to invade the intestinal wall, reach the capillaries and set up foci of infection in the liver and other organs is largely unknown. Two theories have been put forward. One proposes that the amoebae produce a toxin (Councilman & Lafleur, 1891), while the other puts forward the idea that the amoebae secrete a powerful cytolytic enzyme (Dobell, 1919). The toxin theory has received some attention but there is considerable evidence against it (Westphal, 1938). Evidence in favour of the 'cytolytic ferment' theory is derived from the observation that amoebae seen in sections of ulcers are often surrounded by a clear area between the protozoan and adjacent host cells. The clear area is thought to be due to enzymic cytolysis by the amoeba. There is, however, no experimental evidence to support this hypothesis.

Before investigating the relationship of enzyme secretion to the mechanism of tissue invasion, it is necessary to discover the kind of enzymes elaborated by *E. histolytica* and their biochemical characteristics. Other workers have studied the production of proteolytic enzymes by *E. histolytica* by growing amoebae in gelatin solutions (Hallman, Michaelson & DeLamater, 1950; Rees, Baernstein, Reardon & Phillips, 1953; Kaneko, 1956), or placing amoebae upon gelatin films (Harinasuta & Maegraith, 1958), or agar plates containing the substrate (Nakamura & Edwards, 1959*a*; *b*). The methods employed by these workers were qualitative or roughly quantitative. The objective of the present work was to measure the proteolytic activity of different strains of *E. histolytica* employing casein solutions as substrate. A brief note describing the technique and some of the results was published earlier (Neal, 1956).

II. MATERIAL AND METHODS

Biochemical methods. Preliminary attempts to investigate proteolytic enzymes of *E. histolytica* were made with formolized gelatin disks containing powdered charcoal (Kohn, 1953). These were added to cultures of *E. histolytica* grown in the conventional manner, one disk of gelatin per tube. Any hydrolysis of the solid gelatin was detected by the presence of loose charcoal.

Further investigation of the amoebic enzymes was made on extracts prepared from cultures. Amoebae were grown in a medium consisting of solid egg base plus dilute horse-serum liquid overlay and sterile rice starch (*Ehs*+*S*, Dobell &

Laidlaw, 1926). This medium was preferred since higher yields of amoebae were obtained than in other media. The medium was prepared in 2 l. conical flasks with the solid egg base placed horizontally on the base of the flask. In later experiments, flat-sided, screw-capped medicinal bottles were used, with the solid egg base along the flat side of the bottle; they were incubated with the egg base horizontal. The 2 l. conical flasks of *Ehs+S* medium were first seeded with about 1 ml. of a broth culture of *Escherichia coli* (strain A^1) and the bacteria allowed to grow at 37° C. overnight. The medium was then inoculated with about 2×10^6 amoebae in 10–20 ml. The amoebae used for inoculating the large flask were grown in their turn, in the same medium in 100 ml. conical flasks. The yield of amoebae from a 2 l. conical flask incubated at 37° C. for 48 hr. varied with the strain employed, from 80 to 100×10^6 amoebae being obtained. The amoebae were then suspended in the culture supernatant, poured into a suitable glass vessel, centrifuged and washed with an equal volume of Tyrode solution, pH 7·2 to 7·3 (Parker, 1938). After the second centrifuging the amoebae were resuspended in Tyrode solution and the number of amoebae per ml. determined by counting in a haemocytometer. All determinations were an average of four counts. The suspension of amoebae was stored at −20° C. for 16 hr., the resulting lysate constituting the extract. The extract was then thawed and centrifuged to remove rice starch and other debris. As the extract contained a variable number of bacteria it was desirable to inhibit or remove these organisms. Filtration through cellulose acetate membrane filters was tried, but some loss of enzymic activity was always observed (see Fig. 7). Addition of streptomycin and penicillin (100 mg. or units of each antibiotic/ml.) inhibited the bacteria and did not affect proteolytic activity. Addition of a few drops of chloroform to the extract had the same effect. In view of the simplicity of the latter method, it was used throughout this investigation.

Prepared in this manner, the extract was slightly opalescent and free from large amounts of debris, though it contained a variable number of dead or inhibited bacteria. Extracts prepared from the monaxenic strain $P+A^1$ were sterile but those from other strains were not invariably sterile.

For investigating the action of amoebic extracts upon gelatin, a 6% solution of gelatin (Soc. des Produits Chimiques Coignet, fine leaf 'D') in distilled water was tubed in 3 ml. amounts in screw-capped bottles and sterilized by steaming. One batch of gelatin was used throughout the experiments and was stored in the refrigerator at 4° C. When required for use the gelatin was melted at 37° C. and 1 ml. of extract added. The pH was adjusted to 7·8 by the addition of 0·1 N NaOH. An Ostwald viscometer was then filled with the mixture and the whole apparatus immersed in a water bath (37 ± 0·05° C.). The extract and the viscometer were warmed to 37° C. before use. The time taken for the mixture to flow between two points was measured at 15 min. intervals for 1 hr. or at 30 min. intervals for 2 hr.

The hydrolysis of casein was determined by mixing equal volumes of urea–Hammersten casein (prepared according to Duspiva, 1939), amoebic extract and 0·3 M phosphate–ammonia–citric acid buffer to give the required pH. In later experiments the incubation mixture consisted of equal volumes of urea–casein and amoebic extract, the pH being adjusted by addition of 0·1 N NaOH. The final

concentration of casein in the earlier experiments was 1 %, while in the later work the concentration was 1·5 %. The pH of the mixture was always determined with a glass electrode and pH meter. After incubation at 37 ± 0·5° C. in a water-bath, hydrolysis was stopped by adding twice the incubation mixture volume of 0·3 M trichloracetic acid. The tubes were then chilled overnight in the refrigerator (4° C.) and the next day were centrifuged to give a clear supernatant liquid. To 0·2 ml. of supernatant was added 0·8 ml. water, 1·0 ml. 1·0 N NaOH and 0·5 ml. of Folin and Ciocalteau's reagent (diluted 3 times). The intensity of the colour was read in a spectrophotometer at wavelength of 640 mμ. and the tyrosine equivalent determined from a standard curve for tyrosine. This technique is similar to that described by Holter & Løvtrup (1949). Substrate and extract blanks were prepared by precipitation with trichloracetic acid at zero time.

The inhibitory action of sera and culture media was investigated by adding 0·15 ml. of test material to 0·9 ml. mixture of amoebic extract and casein substrate. The pH was adjusted to 7·8. Control blanks were determined at zero incubation time and Tyrode solution was added in place of test material in a second control tube. The tubes were incubated for 2 hr. and the tyrosine equivalent determined. The degree of inhibition is expressed as a percentage of the control tube containing Tyrode solution in place of inhibitor. Trypsin inhibitors were commercial samples of crystalline soy bean inhibitor and partially purified lima bean inhibitor.

The hydrolysis of casein by living amoebae was studied by growing strain $P+A^1$ amoebae in 1 % casein solution. The casein solution was sterilized by Seitz filtration, adjusted to pH 7·0, and tubed in 2 ml. quantities. To each tube, 30×10^3 amoebae were added. After 24, 48 and 72 hr. incubation, the sediment was suspended evenly throughout the liquid, counts of living and dead amoebae were made in duplicate, and trichloracetic acid was added (4 ml. of 0·3 M solution to 2 ml. of casein solution). Tyrosine was then estimated in the manner previously described.

In order to compare the activity of extracts prepared from different strains, it was necessary to allow for the varying concentrations of amoebae from which the extracts were prepared. This was done by calculating the tyrosine equivalent after incubation for 1 hr. from four determinations taken at 15 min. intervals. The calculated figure (T) was then divided by the number of amoebae $\times 10^6$/ml. (N) from which the extract was prepared. This gave the tyrosine equivalent/million amoebae (T/N) or proteolytic index.

Virulence of E. histolytica. Infectivity and invasiveness were determined by inoculation into the caecum of young rats. Invasiveness is represented as the average degree of caecal ulceration (normal caecum scores 0, maximally affected caecum scores 8). Further details of assessment of *E. histolytica* virulence are given by Neal & Vincent (1955) and Neal (1951).

Strains of E. histolytica *and culture media.* As this work has developed intermittently since 1954, a large number of strains of *E. histolytica* have been employed. A monaxenic strain $P+A^1$, growing with *Escherichia coli* (strain A^1) has been used throughout as the reference strain (see Dobell & Neal, 1952 for the history of $P+A^1$). $D+R^4$ was another monaxenic strain used only in experiments with

formolized gelatin. All the remaining strains of amoebae were grown with a mixed unknown bacterial flora. After a settling-down period, all strains grew regularly and profusely, requiring subculturing twice each week. The history of strains *EA*, *ND*, *SI* and *HK*, which were isolated from carriers and were non-invasive to rats, is given by Neal & Vincent (1955). Strain *RD* was attenuated when used in these experiments; its history is given by Neal & Vincent (1956). Strain *JO* was isolated from a carrier and was non-invasive to rats. Five other strains, *LE*, *W*, *OK*, *C* and *CA*, were isolated from patients suffering from amoebic dysentery; the cultured amoebae were invasive to rats.

The *M* series of strains were derived originally from amoebae isolated from an acute case of amoebic dysentery (Neal, 1951). This strain became attenuated by prolonged cultivation *in vitro*. Its virulence was revived by a series of five liver passages in hamsters (see Neal & Vincent, 1956); the recovered amoebae were named *MLVc*. This strain again became attenuated but recovered its virulence after a second series consisting of four liver passages; this gave strain *MALIV*. The strain again lost virulence, which returned after a third series consisting of six passages. This gave strains *MHIV* and *MHVI*, isolated from the fourth and sixth hamster passage, respectively. This series of experiments has been described in detail by Vincent & Neal (1960).

In earlier experiments relating to the proteolytic activity of *E. histolytica*, the bacterial flora were modified so as to eliminate proteolytic bacteria. Strain *M* and *MLVc* amoebae were treated with streptomycin and penicillin and *EA* cysts were treated with acriflavine. The amoebae of each of these strains were subsequently grown with *Escherichia coli* (A^1) and were named *MS*, MA^1 and EA^1, respectively. The elimination of proteolytic bacteria was later found to be unnecessary.

All strains were routinely maintained upon inspissated horse serum plus egg white diluted with Ringer's solution plus rice starch (*HSre* + *S*) and were transferred to *Ehs* + *S* medium for preparation of extracts. Media used for study of inhibitors were egg slant covered with Ringer's solution (*Er*), dilute horse serum with yeast extract (*hsm*, Jones, 1946) and egg yolk infusion (*ys*, Balamuth, 1946).

III. LIQUEFACTION OF GELATIN BY GROWING CULTURES OF *ENTAMOEBA HISTOLYTICA*

Disks of formolized charcoal–gelatin were added to tubes of culture medium and incubated for 4 days with bacteria alone or with amoebae and their concomitant bacteria.

The results (Table 1) showed that the bacterial flora themselves often produced proteases which dissolved gelatin; these bacteria were present in strains which were invasive, attenuated or non-invasive. Where bacteria did not produce proteases, it was possible to test the amoebae for proteolytic activity. Experiments with these strains (MA^1, EA^1, *HK*, *JO* and $P + A^1$) showed that the gelatin disks were dissolved by 4 days incubation at 37° C. The disks were not attacked until the third day and were not completely liquefied until the fourth day. In the case of one strain ($D + R^4$), 7 days' incubation was required to dissolve the gelatin disk.

As the activity was only observed on the third or fourth day, that is when the amoebae started to die, the enzymes responsible were possibly not secreted extracellularly, but diffused from dead amoebae. Attention was therefore directed to extracts prepared from cultured amoebae.

Table 1. *Liquefaction of formolized gelatin disks by cultures of* Entamoeba histolytica

Strains of *E. histolytica* and their virulence to rats		Bacterial flora alone	Bacterial flora + amoebae
MLVc	Invasive	+	−
MA[1]		0	+
M	Attenuated	+	−
RD		+	−
EA	Non-invasive	+	−
EA[1]		0	+
ND		+	−
SI		+	−
HK		0	+
JO		0	+
$P+A^1$	Non-infective	0	+
$D+R^4$		0	+*

* 7 days' incubation. 0 = gelatin not liquefied. + = gelatin liquefied. − = not tested.

IV. THE HYDROLYSIS OF PROTEINS BY EXTRACTS OF *ENTAMOEBA HISTOLYTICA*

The first experiments were designed to determine the optimum conditions for activity and to investigate the properties of the extract.

(1) *Factors affecting determination of activity*

pH. The optimum pH was determined with casein as substrate and extracts of $P+A^1$ amoebae, 3·4 and $3{\cdot}8 \times 10^6$ amoebae/ml. The results (Fig. 1) show that there was high proteolytic activity between pH 5·8 and 8·5, with a peak of activity at pH 7·5 to 7·9. At all pH values, casein hydrolysis due to bacteria alone was negligible (see also below). The variation of activity with pH shown by extracts prepared from invasive amoebae was similar to that shown by extracts prepared from $P+A^1$ amoebae. All subsequent experiments were performed at pH 7·8 to 7·9.

Temperature of incubation. Extracts of $P+A^1$ amoebae ($2{\cdot}1 \times 10^6$ amoebae/ml.) were incubated with casein for 2 hr. at temperatures ranging from 24° to 50° C. Casein hydrolysis proceeded fastest at 37° C., lower or higher temperatures giving lower results. The results in Fig. 2 are expressed in percentages, taking the figure for 37° C. as 100 %.

Incubation time. Extracts were prepared from $P+A^1$ amoebae ($8{\cdot}3 \times 10^6$ amoebae/ml.) and *MALIV* amoebae ($6{\cdot}5 \times 10^6$ amoebae/ml.) and incubated for 6 hr. The results (Fig. 3) show that casein was hydrolysed at a steady rate for

about 2 hr., after which the rate decreased, no further hydrolysis being observed after 3 hr. In the case of *MALIV* extract, the rate of hydrolysis decreased earlier than that with $P+A^1$ extract.

From the results of experiments described so far, greatest activity was observed when the amoebic extract was incubated with casein at pH 7·8 to 7·9 at 37° C.

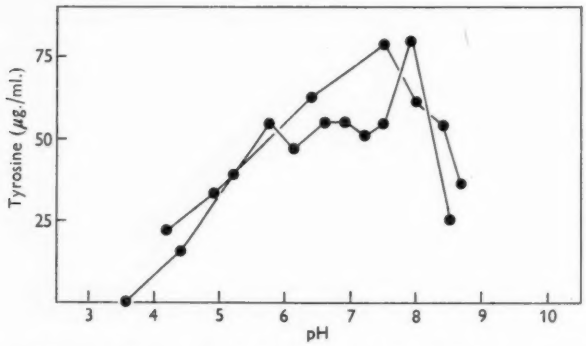


Fig. 1. Effect of pH upon hydrolysis of casein by *E. histolytica* ($P+A^1$).

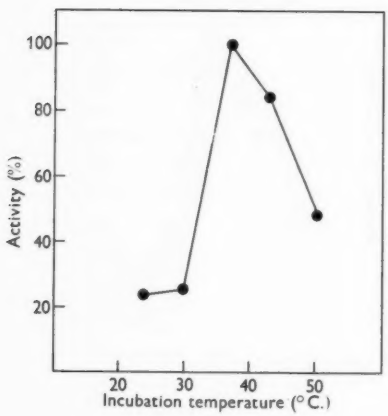


Fig. 2

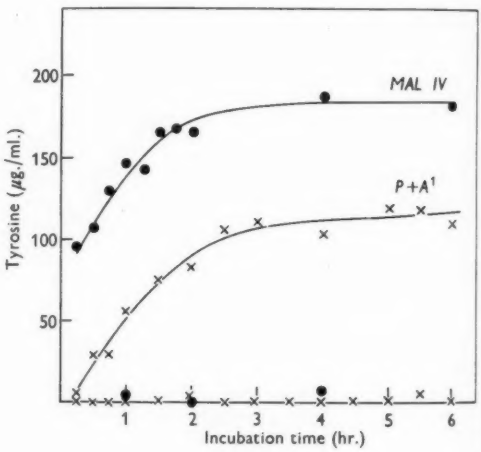


Fig. 3

Fig. 2. Effect of incubation temperature on hydrolysis of casein by *E. histolytica* ($P+A^1$).

Fig. 3. Rate of casein hydrolysis by two strains of *E. histolytica*. The points at bottom of figure are those of bacterial controls.

Under these conditions, hydrolysis ceased after 2 hr. incubation. Unless otherwise stated, these factors were constant in subsequent experiments.

Inactivation. Proteolytic activity was abolished completely by heating the amoebic extract in boiling water for 10 min. or more. The activity was progressively inhibited by heating the extract at 59° C. The results of this experiment are shown

in Fig. 4, expressed as the percentage of activity of the unheated extract. Results with three strains, $P+A^1$, *MALIV* and *SI* (13·3, 4·8 and $5{\cdot}7 \times 10^6$ amoebae/ml., respectively) are illustrated. The addition of cysteine or iodoacetate had no effect on the proteolytic activity.

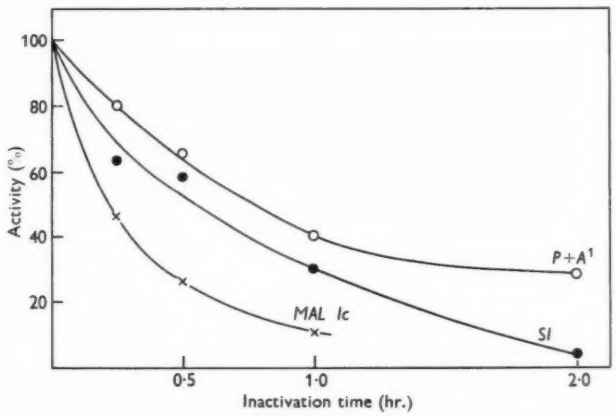


Fig. 4. Inactivation of casein hydrolysis by prior heating of extract at 59° C. Results from three strains of *E. histolytica*.

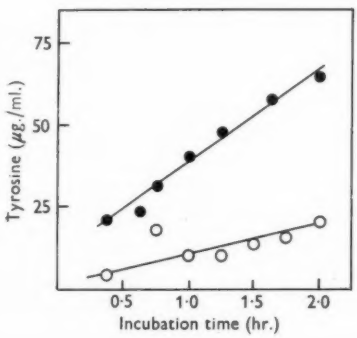


Fig. 5

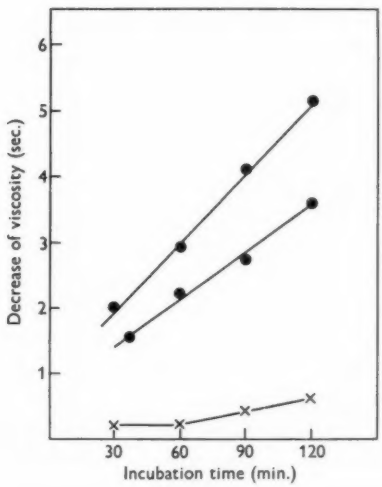


Fig. 6

Fig. 5. Effect of dilution (2×) of extract (strain $P+A^1$) on casein hydrolysis.

Fig. 6. Reduction of gelatin viscosity by dilution of extract (2×) prepared from strain *JO*. Lowest graph represents effect of bacteria alone.

Effect of dilution on activity of amoeba extracts. Amoebic extracts were diluted with an equal volume of Tyrode solution and the activity compared with that of undiluted extracts. An extract prepared from $P+A^1$ amoebae ($4{\cdot}9 \times 10^6$ amoebae/ml.)

was incubated with casein and an extract from *JO* amoebae ($5{\cdot}0 \times 10^6$ amoebae/ml.) incubated with gelatin. From the results (Figs. 5, 6) the slopes of the graphs were calculated. The ratio of the slopes of the undiluted to diluted extracts, instead of the expected 0·50, was found to be 0·32 in the case of the casein experiments and 0·59 for the gelatin experiments.

(2) *Effect of concomitant bacteria upon casein and gelatin*

As all strains of *E. histolytica* used were grown either with known single bacterial species or with an unknown flora, the effect of the bacteria on the substrate was studied. This was especially important since the preliminary study with gelatin-charcoal disks had shown that most cultures of *E. histolytica* contain proteolytic bacteria (see above).

The effect of bacteria was studied in two ways. The first type of experiment involved taking about 10 ml. of the supernatant from the last washing during the preparation of the amoebic extract. This contained only bacteria and was frozen, chloroform added and treated exactly as if it contained amoebae. The bacterial supernatant was then put up with casein or gelatin in parallel with the amoebic extracts. A typical result of the effect of bacterial supernatant upon hydrolysis of casein is shown in Fig. 3. Even after 4 hr. incubation at pH 7·8 the tyrosine equivalent was only 7 μg./ml. with bacteria from *MALIV* which could dissolve formolized gelatin. This is in contrast with the amoebic extract of *MALIV* which gave a value of 194 μg./ml. after 4 hr. incubation. The bacterial extract of $P+A^1$ had even less effect on casein. This was not surprising since *Escherichia coli* (A^1) is not proteolytic (Dobell & Neal, 1952). The effect of bacterial supernatant upon gelatin is shown in Fig. 6. This shows an effect on reduction of viscosity which was negligible compared with the effect of the amoebic extract.

In the amoeba culture, bacterial growth gave large clumps of organisms which were sedimented with the amoebae and rice starch. Therefore the amoebic extract prepared from the sediment contained a different quantity and perhaps different species of bacteria from those present in the supernatant. For this reason the effect of the bacterial flora was investigated by a second method. The flora was separated from the amoebae by leaving cultures in *Ehs* medium at room temperature until the amoebae had died (about 7 days). The absence of amoebae was confirmed by growing the bacteria in *Ehs*+*S* medium. The bacteria was then grown in a 100 ml. flask of *Ehs*+*S* medium for 48 hr., the starch layer collected and an extract prepared just as if amoebae were present. The bacterial extract prepared from the sediment was incubated at pH 7·8 with casein as substrate. The results were similar to those described above for the supernatant extract in that no significant hydrolysis of casein was observed.

In spite of uniformly negative results for bacteria alone, a bacterial extract prepared from the supernatant of the last amoeba washing was always put up in parallel with the amoebic extract. As the bacteria alone always had a negligible effect, bacterial control results are omitted from the figures (except Figs. 3 and 6 already noted).

(3) *Rate of hydrolysis of casein*

The results illustrated in Fig. 3 show that the rate of hydrolysis of casein by extracts prepared from strain $P+A^1$ decreased after about 2 hr. and practically stopped after 3 hr. incubation. The results for strain *MALIV* (also shown in Fig. 3) were similar except that hydrolysis decreased after 1 hr. and stopped after only 2 hr. incubation. An abrupt cessation of hydrolysis was often seen with strains with mixed bacterial floras after 1 hr. of incubation. Since the chloroform treatment did not always sterilize extracts prepared from such strains, it was thought possible that after 1 hr. of incubation the surviving bacteria started to metabolize the smaller molecules which had been split from casein by the amoebic enzyme.

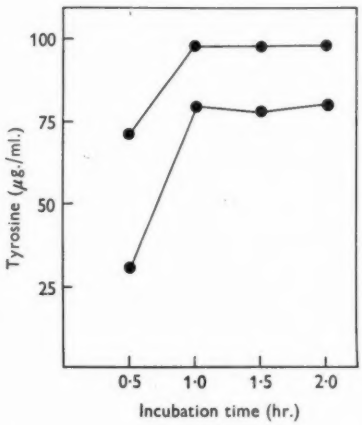


Fig. 7

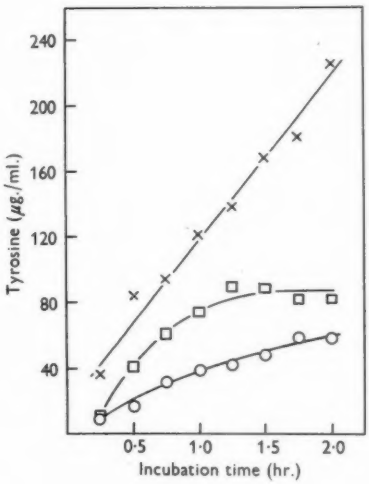


Fig. 8

Fig. 7. Effect of filtration of strain *W* extract through cellulose acetate membrane. Upper graph unfiltered extract, lower graph after filtration.

Fig. 8. Cessation of hydrolysis during incubation with casein (strain *CA*).

The small molecules with phenolic OH-groups would therefore not be available to react with the Folin and Ciocalteau reagent.

This hypothesis was tested by filtering an amoebic extract (strain *W*) through a cellulose acetate membrane, which removed all bacteria, and comparing the activity with that of the unfiltered extract. The results (Fig. 7) show that although filtration reduced the general level of activity, it did not affect the cessation of hydrolysis after 1 hr. incubation.

Further experiments with strain *CA* (Fig. 8) revealed that a highly active and a weak extract (24 and $4{\cdot}7 \times 10^6$ amoebae/ml., respectively) hydrolysed casein at a linear rate, but an extract with intermediate activity (prepared from $9{\cdot}3 \times 10^6$ amoebae/ml.) showed a cessation of enzyme activity after 1 hr. incubation. The less active extracts were obtained by dilution from the most active extract.

This experiment suggests that at a particular concentration of enzymes from *E. histolytica*, an inhibitory factor became operative after about 1 hr. incubation, but above or below this critical level the hydrolysis of casein proceeds at a constant rate over 2 hr. incubation.

(4) *Effect of amoebic extracts upon substrates other than casein and gelatin*

Extracts of amoeba ($P+A^1$) were incubated with potassium hyaluronate (prepared from human umbilical cord), azocoll, egg yolk and haemoglobin prepared by standard methods. The *E. histolytica* extracts did not attack these substances. Haemoglobin was also used as substrate with an extract prepared from an invasive strain (*CA*), but was not attacked. Extracts of invasive amoebae (strain *MHIV*) were incubated with potassium hyaluronate and some degradation was observed. The rate of depolymerization, however, was no greater than that produced by bacteria grown alone and prepared as for amoebae. It was therefore concluded that degradation seen with the amoebic extracts was due to associated bacteria.

(5) *Inhibition of proteolytic activity*

Harinasuta & Maegraith (1958) observed proteolysis of gelatin by amoebae grown in Locke egg-serum medium only when the amoebae were washed. This, they considered, was due to the presence of serum and to its removal on washing.

The inhibitory effect of serum was studied further in the present experiments (Table 2). The sera of all animals studied (horse, rat, cat and man) were inhibitory.

The activity of horse serum was reduced by dilution of the serum, but heating for 65 min. at 56° C. did not reduce the inhibiting effect. The inhibitory activity of rat serum taken from animals infected with *E. histolytica* was similar to that of normal rats; further, the presence or absence of caecal ulceration caused by *E. histolytica* did not affect the inhibitory power of rat serum.

All the above experiments were made with casein as substrate, but an experiment was also performed with gelatin as substrate. In this experiment, 0·33 ml. of Tyrode solution or serum was added to 0·67 ml. of amoebic (strain *MALIV* 4×10^6 amoebae/ml.) or bacterial extract, briefly shaken, then the mixture added to 3 ml. of 6 % gelatin. The pH was adjusted to 7·7. The results (Fig. 9) show that the addition of serum completely inhibits the reduction of viscosity by the amoebic extract. The residual effect was not distinguishable from that of the bacterial extract.

As the usual culture media employed for maintenance of strains of *E. histolytica* often contain serum, their inhibitory power was examined with casein as substrate. It was found (Table 2) that *Ehs*, *Er* and *ys* media showed inhibitory action while *HSre* and *hsm* were not inhibitory. The previous results had shown that two-fold dilution of horse serum reduced the inhibition by approximately 50 %. The horse serum used for overlay in *Ehs* medium is diluted 1:8, which should therefore show about 10 % inhibition. But *Ehs* overlay showed 44 % inhibition, while *Er* and *ys*, which do not contain horse serum, showed greater inhibition (64 and 74 %, respectively). Therefore the egg component appears to contain an inhibitor. This conclusion was confirmed by preparing an extract from egg slopes as prepared for

Table 2. *The effect of various substances as inhibitors of proteolytic activity*

Substance	Degree of inhibition (%) To extracts of $P+A^1$	To extracts of MA^1
Sera		
Horse	90	82
Horse (diluted 1:1)	—	47
Horse (inactivated)	—	91
Normal rat	—	95
Rat *a**	78	—
Rat *b*†	80	—
Cat	77	—
Man	71	—
Culture media		
Sterile *Ehs* overlay	44	—
Sterile *HSre* overlay	3	—
Sterile *Er* overlay	64	—
Sterile *hsm*	0	—
Sterile *ys*	74	—
Solid egg extract	47	—
Ehs + *Escherichia coli* (A^1)	45	—
HSre + *Escherichia coli* (A^1)	0	—
Purified trypsin inhibitors		
Soy bean	27	0
Lima bean	0	0

* = serum from rat infected with *E. histolytica*, without caecal ulceration.
† = serum from infected rat with caecal ulceration.

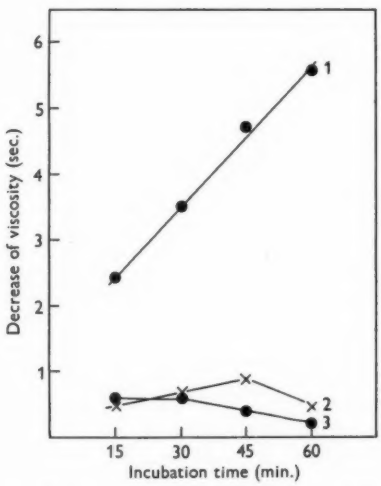


Fig. 9. Inhibitory effect of horse serum on gelatin hydrolysis by *E. histolytica* extract (strain *MALIV*). 1. Amoeba extract. 2. Bacteria extract. 3. Amoeba extract + horse serum.

culture medium. Twenty-five grams of egg slope were blended with 20 ml. distilled water, kept overnight in the refrigerator, and centrifuged. The supernatant was tested for inhibition and was found to be equal to that of the overlay from *Ehs* medium. Growth of *Escherichia coli* (strain A^1) in *Ehs* for 24 hr. did not diminish the inhibition.

As the amoeba proteinase resembles trypsin, and serum also contains a trypsin inhibitor, the effect of trypsin inhibitors from soy bean and lima bean was investigated (see Laskowski & Laskowski, 1954, for general review on trypsin inhibitors). It was first determined that 111 μg./ml. of crystalline soy bean inhibitor or crude lima bean inhibitor completely prevented the hydrolysis of casein by trypsin solution containing 3·3 μg./ml. of crystalline trypsin. This trypsin concentration gave an activity equivalent to an amoebic extract prepared from 8×10^6 amoebae/ml. Soy and lima bean trypsin inhibitors were then added to casein and amoebic extract mixture in the concentration of 143 μg./ml. The results (Table 2) show that the trypsin inhibitors were without effect upon the amoeba enzyme.

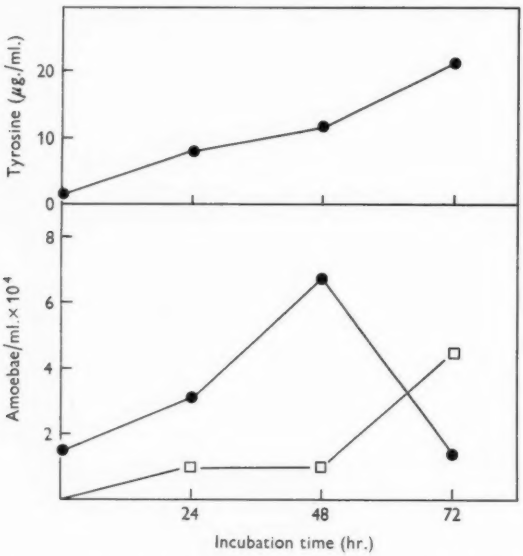


Fig. 10. Growth of *E. histolytica* ($P+A^1$) in casein solution. Upper graph shows tyrosine equivalent, lower graph shows numbers of living amoebae (—●—) and dead amoebae (—□—).

V. ATTEMPTS TO DETECT EXTRACELLULAR PROTEOLYTIC ENZYMES

The results described above show that gelatin disks placed in tubes of *E. histolytica* cultures were hydrolysed after about 3 days' incubation at 37° C. This could have been due to secretion of proteolytic enzymes into the culture medium or to intracellular enzymes released into the medium on the death of the amoebae. Further investigation of these possibilities was made.

Incubation of the medium after growth of *E. histolytica* ($P+A^1$) for 48 hr. with casein did not show any protease activity, so attempts were made to concentrate any undetected low activity. General protein concentration techniques were employed with the liquid phase of culture media after 48 hr. growth of *E. histolytica* strain $P+A^1$. Acetone (50 %, vol./vol.) was added to 100 ml. of *Ehs* culture liquid, the resulting precipitate being washed and resuspended in 20 ml. Sørenson phosphate buffer pH 7·2. In a second experiment, 100 ml. of *HSre* culture liquid were treated with ammonium sulphate to give a saturated solution. The precipitate was redissolved in 5 ml. Sørenson's buffer. In neither case was there any hydrolysis of casein by the concentrates.

A further experiment was made by growing amoebae (strain $P+A^1$) in 0·5 % casein solution, testing the solution for hydrolysis with Folin and Ciocalteau's reagent in the usual manner after 24, 48 and 72 hr. incubation, and correlating these results with the number of live and dead amoebae/ml. The results (Fig. 10, an average of two experiments) show that there was some hydrolysis of casein up to 48 hr. and that the rate of hydrolysis was increased between 48 and 72 hr. The amoebae did not grow well in the casein solution; some dead amoebae were present at each examination, but they started to die in large numbers after 48 hr. The slowest rate of casein hydrolysis occurred when the amoebae were multiplying (24–48 hr.), while the fastest rate occurred when the amoebae were dying (48–72 hr.).

VI. RELATIONSHIP BETWEEN PROTEOLYTIC ACTIVITY AND INVASIVENESS OF *ENTAMOEBA HISTOLYTICA*

The activity of the monaxenic strain of *E. histolytica* $P+A^1$ was first studied. This showed (Table 3) that in six experiments the estimate of proteolytic activity (or proteolytic index, T/N) varied between 4·7 and 10·6 (average 7·70).

Table 3. *Estimate of proteolytic activity of* Entamoeba histolytica *strain* $P+A^1$

Tyrosine (μg./ml.) after 1 hr. incubation (T)	No. of amoebae (million/ml.) in extract (N)	Proteolytic index (T/N)
40	4·9	8·2
50	6·9	7·3
54	5·1	10·6
46	8·3	5·5
34	7·3	4·7
80	8·0	10·0

A series of strains isolated from man were next investigated (Table 4). Strains EA^1 and *SI* from symptomless carriers had been maintained in culture for several years. These strains were not invasive to rats and gave a low proteolytic index. The remaining strains in Table 4 were isolated from patients with symptoms, and all strains were invasive to rats. Strains *C* and *CA*, however, were less infective than the other strains. The proteolytic indices of these strains varied from low

figures for strains *OK*, *C* and *CA* to high figures for strains *LE* and *W*. The low indices could not be explained by prolonged cultivation *in vitro* since they were tested within 12 weeks of isolation. A portion of each amoeba suspension used for preparation of an extract was inoculated into young rats. These experiments showed that the amoebae were still invasive.

Another series of experiments was performed with strain *M* before and after the restoration of virulence by liver passage in the hamster. The results (Table 5)

Table 4. *Proteolytic activity of invasive and non-invasive strains of* Entamoeba histolytica

Strain	Duration of *in vitro* cultivation (weeks)	Invasiveness to rats: No. infected / No. inoculated	Invasiveness to rats: Degree of ulceration of infected rats	Proteolytic index (T/N)
EA[1]	198	9/10	0·67	2·1
SI	165	8/12	1·2	10·0
LE	3	8/8	5·5	18·1
W	3	10/10	6·7	26·4
	12	—	—	22·3
OK	8	10/10	5·1	9·0
	23	—	—	12·2
C	7	5/10	4·8	12·3
CA	12	9/12	5·1	7·4
	16	—	—	8·5

Table 5. *Proteolytic activity of* Entamoeba histolytica *strain* M *before and after restoration of invasiveness by liver passage*

Liver passage	Strain	Duration of *in vitro* cultivation (weeks)	Invasiveness to rats: No. infected / No. inoculated	Invasiveness to rats: Degree of ulceration of infected rats	Proteolytic index (T/N)
Before liver passage	*MS*	457	—	—	3·1
passage		463	1/10	2·0	4·6
After 1st series of passages	*MA*[1]	111	8/8	6·3	20·4
		121	6/7	3·3	6·3
		125	8/8	2·8	12·5
		134	4/7	1·9	9·8
After 2nd series of passages	*MALIV*	4	6/7	5·1	22·2
		9	14/14	4·9	17·1
		12	12/12	3·3	8·2
		67	6/10*	3·5*	7·3
After 3rd series of passages	*MHIV*	4	10/10*	5·2*	6·5†
		7	6/8*	6·3*	5·6
	MHVI	1	6/6*	5·2*	6·0†
		3	4/4*	6·6*	5·6

* These figures are for virulence obtained with amoebae inoculated into rats 7 days before and after the date of the enzyme experiments.

† These experiments were performed simultaneously with amoebae grown in *HSre+S*.

show that after the first series of liver passages the proteolytic index rose from 4·6 to 20·4 (MA^1); this was accompanied by an increase in virulence to rats. After MA^1 had been maintained *in vitro* for 121 weeks, the proteolytic index dropped to 6·3 and continued at a low level in later experiments. A loss of invasiveness was observed at the same time. A second series of liver passages was made and for a second time the invasiveness and proteolytic index increased after passage (strain *MALIV*). However, a third series of passages with attenuated *MALIV* did not restore the proteolytic index, but it did raise the invasiveness to rats. In the case of *M* amoebae isolated after the first liver series (strain MA^1), the high proteolytic index was found after 111 weeks of *in vitro* cultivation but not after 121 weeks or longer periods. The high proteolytic index of *M* amoebae isolated after the second liver series (strain *MALIV*) was observed after 9 weeks' cultivation *in vitro* but disappeared after 12 weeks.

VII. DISCUSSION

The results extend those previously published by other workers. Hallman *et al.* (1950); Rees *et al.* (1953) and Kaneko (1956) showed that growing cultures of *E. histolytica* liquefied gelatin. Harinasuta & Maegraith (1958) placed living amoebae upon the gelatin emulsion of photographic film, while Nakamura & Edwards (1959*a*, *b*) used agar plates containing the substrate (casein or gelatin) for demonstration of protein hydrolysis. Harinasuta & Maegraith (1958) and Nakamura & Edwards (1959*a*, *b*) found proteolytic activity in all strains of *E. histolytica* examined, this activity occurring within the pH range of 5·0 to 8·0. The present results obtained with sensitive quantitative methods, show that there is a peak of activity between pH 7·5 and 7·9. The narrow range of 7·9 to 8·0 reported previously (Neal, 1956) is not correct.

Tests for proteolytic activity on gelatin-charcoal disks showed that the majority of strains of *E. histolytica* contained proteolytic bacteria, except when the flora had been deliberately changed. However, bacterial extracts prepared in the same manner as for amoebae did not hydrolyse casein or gelatin and it was therefore possible to investigate the nature of the hydrolytic action of amoebic extract.

The sensitivity to pH, thermolability, and reduction of activity by dilution, shows that the activity of the amoebic extract is due to the presence of an enzyme. This enzyme can attack casein and gelatin, but not haemoglobin. As activity is not increased by the addition of cysteine nor decreased by iodoacetate, the enzyme does not appear to require sulphydryl groups for activation. The rate of hydrolysis is complicated by the presence of an inhibitory factor observed with extracts of some strains of *E. histolytica*, which seems to operate after about 1 hr. incubation. The nature of this inhibition is unknown. Before further identification of the amoebic proteinase can be made, work with synthetic peptide substrates will be necessary. As the material under investigation is produced by extraction of cells of *E. histolytica* it is most likely to contain peptidase as well as proteinase. The tyrosine equivalent, therefore, is probably the end result of a chain of reactions

involving a number of proteases, but the optimal conditions described refer to the proteinase which starts the reaction.

The absence of hyaluronidase in *E. histolytica* agrees with the observations of Townshend (1949) and DeLamater, Michaelson, Hallman & Blumenthal (1954) but is contrary to Bradin's (1953) results. It is important to note that in the present work the bacterial control prepared from the supernantant of the last amoeba washing did not show any action upon potassium hyaluronate, but the extract prepared from bacteria alone did depolymerize the substrate. As egg-yolk extract and azocoll were unaffected by the amoebic extract, *E. histolytica* does not contain lecithinase or collagenase. The absence of collagenase was also noted by Nakamura & Klein (1957). One of the surprising observations is that the *E. histolytica* proteinase did not attack haemoglobin *in vitro*, as amoebae will ingest and apparently digest large numbers of red blood cells, if these are available.

The experiments on the inhibitory effect of sera confirms the work of Harinasuta & Maegraith (1958). The serum inhibitor was present in serum from all the animals tested, including man, and was not affected by heating to 56° C. for 30 min. The inhibitory power of horse serum was destroyed by inspissation (80° C. for 90 min.), since the liquid part of *HSre* medium was not inhibitory. The inhibitory level of sera did not vary according to the susceptibility of the animal to infection with *E. histolytica*. The liquid part of culture media prepared from hens' eggs was also inhibitory. This was not due to horse serum used in the preparation of the medium, as the serum was diluted to a level at which its inhibitory action would not be detectable. Further, the egg medium inhibition was present in media not containing serum. An inhibitor could also be extracted from whole eggs solidified by heat. It is likely that the egg and serum inhibitors are different substances. The serum inhibitor is probably a protein, perhaps similar to the trypsin inhibitor, while the egg inhibitor is a substance of smaller molecular size and not of a protein nature. Since *ys* medium is inhibitory, it must be present in the egg yolk rather than in the egg white.

The results of experiments described above indicate that proteinase was not secreted into the culture medium, and that such hydrolysis of substrate as did occur could be explained by the release of enzyme from dead amoebic cells. On the other hand, Harinasuta & Maegraith (1958) found that the gelatin emulsion of 35 mm. photographic film was digested within 1 hr. by a suspension of living amoebae. They examined the suspension after 1 hr. incubation and found the amoebae 'to be still alive'. However, it is not stated if any of the amoebae had died, since a small proportion of dead amoebae could release enough enzyme to digest the gelatin layer. Harinasuta & Maegraith also found that the supernatant from a suspension of amoebae after incubation in buffered saline for 2 hr. did not hydrolyse gelatin. A possible explanation of these discrepancies is given below.

The presence of inhibitor in culture media might affect the invasiveness or the detection of proteolytic activity. However, Thompson, McCarthy & Reinertson (1954), who used a *Locke–egg–serum* medium for some of their work, reported a decline of virulence similar to that observed with amoebae maintained in *HSre + S* (Vincent & Neal, 1960). The *Locke–egg–serum* medium is presumably inhibitory

by virtue of the egg component, while *HSre+S* as shown above is not inhibitory. However, the whole relationship between type of culture medium, invasiveness and proteolytic activity is worthy of re-examination.

Turning to the correlation of proteolytic activity and invasiveness of different strains of *E. histolytica*, the activity of strain $P+A^1$ in six experiments gave a proteolytic index which varied between 4·7 and 10·6. This is considered to be the level for general metabolic activity of *E. histolytica*. Two non-invasive strains, established *in vitro* for several years, had similar low proteolytic indices.

The rise of virulence of *M* strains after liver passage was accompanied on two occasions by a rise of the proteolytic index but not on a third occasion. In connexion with these results, the duration of invasiveness after liver passage (Vincent & Neal, 1960) was compared with the duration of high proteolytic activity (Table 6). This shows a progressive decrease in the duration of both invasiveness and proteolytic activity, the high proteolytic activity disappearing before invasiveness. The decline in the duration of high proteolytic activity

Table 6. *Duration of invasiveness compared with the duration of high proteolytic activity of strain* M *after three series of liver passages*

No. of the series of liver passage	Duration of invasiveness (weeks)*	Duration of high proteolytic activity (weeks)
1	183	111
2	69	9
3	45	0

* Figures from Vincent & Neal (1960).

therefore appears to act as a measure of declining virulence of *E. histolytica*. The discrepancy between time of disappearance of invasiveness and high proteolytic activity was 72 and 60 weeks for the first and second series, respectively. The duration of invasiveness after the third series of passages was only 45 weeks and it was therefore not surprising to find only low proteolytic activity. Two possible explanations for the time lag are that either there may be a period when the proteases are not detectable *in vitro*, but may still be revived by the animal environment, or high proteolytic activity may not be required for tissue penetration but merely accompanies another factor. Even if it is not the sole factor the presence of higher proteolytic activity in virulent amoebae is readily explained, since amoebae living in tissue would have greater amounts of protein available than those living in the gut lumen.

The results obtained using freshly isolated strains from patients with diarrhoea and dysentery were not consistent. Two strains (*LE* and *W*) showed high activity, while the other three (*OK*, *C* and *CA*) had low activity. This tends to confirm the conclusion that high proteolytic activity accompanies another factor necessary for invasiveness, which is also supported by the absence of extra cellular enzyme *in vitro* and by the potency of the serum inhibitor.

However, there is a further aspect to this problem. It is now well known that

the cell membrane is not an inert lipoprotein layer but contains enzymes (cf. Mitchell, 1958). Rothstein (1954) considered that hyaluronidase is located at the surface of bacterial cells and Morrison & Neurath (1953) found proteolytic activity in the stroma of human red cells. During excystation of all *histolytica*-like species which have so far been studied, *E. histolytica* (Dobell, 1928), *E. invadens* (Geiman & Ratcliffe, 1936), *E. aulastomi* (Bishop, 1937) and *E. moshkovskii* (Neal, 1953), the hatching amoeba repeatedly strokes a portion of the cyst wall with a pseudopodium. It subsequently hatches through a minute hole. It is likely that this escape hole is produced by the action of enzymes located in or upon the cell membrane of the amoeba. It is therefore not impossible that proteolysis may be effected by enzymes similarly present in the cell membrane. If this is possible the mobility of the cell membrane will ensure effective contact with the substrate. The location of proteinase in the cell wall may also protect the enzyme from the serum inhibitor. It is possible therefore that proteolytic activity may be more accurately measured by allowing living amoebae to attack a solid surface such as a gelatin film or agar containing the substrate. Such methods have been already described (see p. 531) for qualitative observations.

VIII. SUMMARY

The proteolytic activity of extracts of *Entamoeba histolytica* has been further investigated. Casein and gelatin, but not haemaglobin, were hydrolysed. Activity was observed in the pH range 5·8 to 8·5 with optimal activity at pH 7·5 to 7·9. Activity was optimal at 37° C. and sulphydryl groups were not required. Concomitant bacteria showed no proteolytic activity. Hyaluronidase, collagenase and lecithinase could not be detected.

An inhibitor of proteolytic enzyme was present in sera of all animals tested and in egg yolk. All culture media prepared from eggs were inhibitory, but inspissation or dilution of serum inactivated the serum inhibitor. Purified trypsin inhibitors from lima and soy bean were not active against the amoebic enzyme. Proteolytic enzymes were not secreted extracellularly *in vitro*.

High proteolytic activity was found in two out of five invasive, freshly isolated, strains of *E. histolytica* and after two series of liver passages of a single strain. The significance of these observations is discussed. It is concluded that the present evidence does not convincingly demonstrate that high proteolytic activity is required for tissue invasion by amoebae, but may accompany another factor.

I would like to thank Dr R. H. Nimmo-Smith for performing the hyaluronidase tests. Mr E. W. Rogers and Mrs R. Legg for assistance with tyrosine estimations, and Miss S. A. Stewart for help with amoeba cultivation and manipulation. Mr A. V. Allen of the Hospital for Tropical Diseases kindly supplied the material for isolation of new strains of *E. histolytica*.

REFERENCES

BALAMUTH, W. (1946). Improved egg yolk infusion for cultivation of *Entamoeba histolytica* and other intestinal protozoa. *Amer. J. Clin. Path.* **16**, 380.

BISHOP, A. (1937). Further observations upon *Entamoeba aulastomi* Nöller. *Parasitology*, **29**, 57.

BRADIN, J. R. (1953). Studies on the production of hyaluronidase by *Endamoeba histolytica*. *Exp. Parasit.* **2**, 230.

COUNCILMAN, W. T. & LAFLEUR, H. A. (1891). Amoebic dysentery. *Johns Hopk. Hosp. Rep.* **2**, 395.

DELAMATER, J. N., MICHAELSON, J. B., HALLMAN, F. A. & BLUMENTHAL, H. (1954). An investigation into hyaluronidase as a factor in the mechanism of tissue invasion by *Endamoeba histolytica*. *Amer. J. Trop. Med. Hyg.* **3**, 1.

DOBELL, C. (1919). *The Amoebae Living in Man*. London: John Bale, Sons and Danielsson.

DOBELL, C. (1928). Researches on the intestinal protozoa of monkeys and man. I. General introduction and II. Description of the whole life history of *Entamoeba histolytica* in cultures. *Parasitology*, **20**, 357.

DOBELL, C. & LAIDLAW, P. P. (1926). On the cultivation of *Entamoeba histolytica* and some other entozoic amoebae. *Parasitology*, **18**, 283.

DOBELL, C. & NEAL, R. A. (1952). Research on the intestinal protozoa of monkeys and man. XII. Bacterial factors influencing the life history of *Entamoeba histolytica* in cultures. *Parasitology*, **42**, 16.

DUSPIVA, F. (1939). Beiträge zur Histophysiologie des Insektendarmes. I. Untersuchungen über die Verteilung der proteolytischen Enzyme sowie der sekret- und resorptionszellen in Darm von *Dytiscus marginalis*. *Protoplasma*, **32**, 211.

GEIMAN, Q. M. & RATCLIFFE, H. L. (1936). Morphology and life-cycle of an amoeba producing amoebiasis in reptiles. *Parasitology*, **28**, 208.

HALLMAN, F. A., MICHAELSON, J. B. & DELAMATER, J. N. (1950). The cultivation of *Endamoeba histolytica* in a defined medium. *Amer. J. Trop. Med.* **30**, 363.

HARINASUTA, C. & MAEGRAITH, B. G. (1958). The demonstration of proteolytic enzyme activity of *Entamoeba histolytica* by the use of photographic gelatin film. *Ann. Trop. Med. Parasit.* **52**, 508.

HOLTER, H. & LØVTRUP, S. (1949). Proteolytic enzymes in *Chaos chaos*. *C.R. Lab. Carlsberg*, Sér. chim. **27**, 27.

JONES, W. R. (1946). The experimental infection of rats with *Entamoeba histolytica*; with a method for evaluating the anti-amoebic properties of new compounds. *Ann. Trop. Med. Parasit.* **40**, 130.

KANEKO, M. (1956). Investigation on the proteolytic activity of parasitic and free-living amoebae. *Kiseichu-gaku Zasshi*, **5**, 92.

KOHN, J. (1953). A preliminary report of a new gelatin liquefaction method. *J. Clin. Path.* **6**, 249.

LASKOWSKI, M. & LASKOWSKI, M. JR. (1954). Naturally occurring trypsin inhibitors. *Advanc. Protein Chem.* **9**, 203.

MITCHELL, P. (1958). Membrane penetration and therapeutic value of chemicals. In *Strategy of Chemotherapy*. *Symp. Soc. Gen. Microbiol.* **8**, 94.

MORRISON, W. L. & NEURATH, H. (1953). Proteolytic enzymes of the formed elements of human blood. I. Erythrocytes. *J. Biol. Chem.* **200**, 39.

NAKAMURA, M. & EDWARDS, P. R. Jr. (1959*a*). Casease in *Entamoeba histolytica*. *Nature, Lond.*, **183**, 397.

NAKAMURA, M. & EDWARDS, P. R. Jr. (1959*b*). Enzymes of *Entamoeba histolytica*. I. Gelatinase. *Proc. Soc. Exp. Biol. N.Y.*, **100**, 403.

NAKAMURA, M. & KLEIN, L. (1957). Absence of collagenase activity in *Entamoeba histolytica*. *J. Parasit.* **43**, 88.

NEAL, R. A. (1951). Some observations on the variation of virulence and response to chemotherapy of strains of *Entamoeba histolytica* in rats. *Trans. Roy. Soc. Trop. Med. Hyg.* **44**, 439.

NEAL, R. A. (1953). Studies on the morphology and biology of *Entamoeba moshkovskii* Tshalaia, 1941. *Parasitology*, **43**, 253.

NEAL, R. A. (1956). Proteolytic enzymes in *Entamoeba histolytica*. *Nature, Lond.*, **178**, 599.

NEAL, R. A. (1958). The pathogenicity of *Entamoeba histolytica*. *Proc. 6th Int. Congr. Trop. Med. Malaria*. In the Press.

NEAL, R. A. & VINCENT, P. (1955). Strain variation in *Entamoeba histolytica*. I. Correlation of invasiveness in rats with the clinical history and treatment of the experimental infections. *Parasitology*, **45**, 152.

NEAL, R. A. & VINCENT, P. (1956). Strain variation in *Entamoeba histolytica*. II. The effect of serial liver passage on the virulence. *Parasitology*, **46**, 173.

PARKER, R. C. (1938). *Methods of Tissue Culture*, p. 36. London: Hamish Hamilton.

REES, C. W., BAERNSTEIN, H. D., REARDON, L. V. & PHILLIPS, L. (1953). Some interactions *in vitro* of *Endamoeba histolytica* and single species of microbial symbionts. *Amer. J. Trop. Med. Hyg.* **2**, 1002.

ROTHSTEIN, A. (1954). The enzymology of the cell surface. In *Protoplasmatologia*, **2**, (E4) 1. Wien: Springer-Verlag.

THOMPSON, P. E., MCCARTHY, D. & REINERTSON, J. W. (1954). Observations on the virulence of *Endamoeba histolytica* during prolonged subcultivation. *Amer. J. Hyg.* **59**, 249.

TOWNSHEND, R. H. (1949). Failure of an attempt to demonstrate hyaluronidase production by *Entamoeba histolytica*. *Trans. Roy. Soc. Trop. Med. Hyg.* **42**, 314.

VINCENT, P. & NEAL, R. A. (1960). Duration of invasiveness of *Entamoeba histolytica* maintained *in vitro*. *Parasitology*. **50**, 449.

WESTPHAL, A. (1938). Die Pathogenese der Amöbenruhr bei Mensch und Tier. I. Das Wesen der pathogenetischen Wirksamkeit der Ruhramöbe. *Arch. Schiffs-u. Tropenhyg.* **42**, 343.

Parasitology (1960), **50**, 551–575
With 8 text-figures
Printed in Great Britain

Trematode parasites of snails from Edgbaston Pool: the life history of the strigeid *Cotylurus brevis* Dubois & Rausch, 1950

By P. NASIR, Ph.D.*
Department of Zoology and Comparative Physiology, University of Birmingham

(*Received* 28 *September* 1959)

For a number of years it has been the practice in the Zoology Department at the University of Birmingham to provide living larval trematodes, readily obtained from snails from the nearby Edgbaston Pool, as demonstration material for undergraduate classes. The material used, however, has never been accurately identified, since a search of the literature revealed that very little work on larval trematodes has been done in Britain: to date there have been papers from no more than half a dozen workers on fresh-water forms, and from a lesser number on marine forms. Even among this small group of workers there have been very few attempts to investigate complete life cycles of trematodes, and the work of Harper (1929, 1931) and of Rees (1932, 1939, 1940, 1952, 1955, 1957) is outstanding. Some work on larval trematodes of the Birmingham area had been carried out by Brown (1926), but his snails were obtained from localities other than Edgbaston Pool and his papers proved to be inadequate for the identification of most of the larvae in snails from Edgbaston Pool.

So far twelve species of cercariae have been found: six echinostomes, three xiphidocercariae, two strigeid cercariae and one schistosome. The present paper deals with the complete life history of one of these, namely, a non-ocellate, pharyngeate, longifurcate cercaria from *Lymnaea stagnalis* (L.), which has been found to develop into the adult strigeid *Cotylurus brevis* Dubois & Rausch, 1950.

C. brevis was first described by Dubois & Rausch (1950) from the intestine of the Pochard Duck *Aythya affinis* from Michigan, North America. The parasite has been found again in Switzerland (Dubois, 1948) in the Eider Duck *Somateria mollissima*, in *Aythya fuligula* and in *A. marila* (in the collections of André, Fuhrmann and Baer). The examination of various specimens (Berlin, Upsala, Bucharest and Sprehn's collection) convinced Dubois and Rausch that *Cotylurus brevis* had often been confused with *C. cornutus* (Rudolphi).

Dubois & Rausch (1950) hinted that the larva of *C. brevis* might be *Cercaria helvetica XXXIV*, which Dubois (1934) had described from *Lymnaea stagnalis* from Neuchâtel. Later Dubois (1953), being of the opinion that the adult '*Strigea tarda*', of which the life cycle has been described by Mathias (1925), was identical with *Cotylurus brevis*, assumed that the life cycle of *C. brevis* was known. '*Cercaria strigea tardae*' from which Mathias's adult specimens were obtained was described

* Present address: Parasitology Laboratory, Wayne State University, Detroit, Michigan, U.S.A.

by Mathias as having fourteen flame cells (an observation that was confirmed by Harper, 1931 and by Wesenberg-Lund, 1934), whereas in Dubois's '*Cercaria helvetica XXXIV*' (and in the cercaria from Edgbaston Pool) the number of flame cells is twenty. Moreover, Harper's specimens of the fourteen flame-celled '*Cercaria A* Szidat' had been bred out into adults that were identified by Dr Baylis of the British Museum as *Cotylurus cornutus*, though this, of course, was before Dubois & Rausch had proposed the separation of *C. brevis* from *C. cornutus*.

Thus for the accurate identification of the fork-tailed cercaria from *Lymnaea stagnalis* from Edgbaston Pool (provisionally identified as *Cercaria helvetica XXXIV*, Dubois, 1934), it seemed desirable that the cercaria be reared to the adult stage. This became essential when I came to know of the existence of another very similar cercaria. By the kind co-operation of Dr Erasmus and Dr Iles of Cardiff, I was able to compare living specimens of the fork-tailed cercaria from *Lymnaea stagnalis* from Edgbaston Pool with living specimens of a cercaria from *Planorbis corneus* from Cardiff described by Dr Iles (1959). I have found it impossible to distinguish these two cercariae from each other morphologically, but there is a well-established biological difference: the cercaria from Edgbaston Pool forms tetracotyle larvae in snails but not in leeches, but the one from Cardiff forms tetracotyle larvae in leeches but not in snails.

In these circumstances the complete life history of *Cotylurus brevis* from cercariae in *Lymnaea stagnalis* from Edgbaston Pool has been investigated in detail, using laboratory-bred second intermediate and definitive hosts.

MATERIAL AND TECHNIQUES

Edgbaston Pool is a permanent artificial triangular-shaped lake of about 2 acres, bordered on its western banks by a mixed wood which is a protected bird sanctuary kept under observation by members of the Birmingham Natural History Society, on the east by a golf course, and on the south by an artificial dam.

I am very grateful to Dr Brian Mitchell of this department for supplying me with the following list (Table 1) of birds (among which the natural definitive hosts of some of the water-snail parasites are likely to be found), which he observed to frequent Edgbaston Pool during the period 1954–56. These birds were identified according to Peterson, Montfoot & Hallom (1954), and named as in the *List of British Vertebrates* published by the British Museum of Natural History in 1935.

There are four common species of pond snail in the lake: *Lymnaea stagnalis* (L.), *L. pereger* (O. F. Müll.), *L. auricularia* (L.) and *Planorbis carinatus* (O. F. Müll.). A detailed examination has been made of large samples (1000–4000) of three of these species of snails, but of a smaller sample (200–300) of *L. pereger*. Some other gastropods occur much more rarely, namely *L. palustris* (Müll.), *P. corneus* (L.) and *P. complanatus* (L.), but these have not been examined for parasites.

Specimens of *Lymnaea stagnalis* collected from the pond were kept in aquaria at 19°–24° C., and fed on either fresh or dried and powdered lettuce leaves. Snail eggs were collected from these aquaria so as to set up separate cultures of

laboratory-bred snails to be available for experimental infections. Eggs hatched in about 11–21 days after being laid, and these snails themselves commenced laying eggs about 8 weeks later. The breeding colonies of snails were maintained uninterruptedly for about 4 years.

In order to identify infected snails, the snails brought into the laboratory from the pool were isolated in sample tubes of water for about 48 hr; the presence of free-swimming cercariae in these tubes indicated which were the infected snails. The shells of such snails were marked with a waterproof ink, and the snails were maintained in aquaria, thus providing ready supplies of cercariae, when required, for experiments.

Table 1. '*Waterbirds*' *seen at Edgbaston Pool*, 1954 *to* 1956

Colymbiformes Podicipidae		
Podiceps cristatus cristatus (L.)	Great crested grebe	Very common
P. ruficollis ruficollis (Pallas)	Little grebe or dabchick	Very common
Ciconiiformes Ardeidae		
Ardea cinerea cinerea, L.	Heron	Common
Anseriformes Anatidae		
Anas clypeata (L.)	Common shoveller	Less common
Anas platyrhynchaos platyrhynchaos, L.	Mallard	Very common
A. crecca crecca (L.)	European greenwinged teal	Very common
A. penelope (L.)	European wigeon	Less common
Aythya fuligula, L.	Tufted duck	Very common
A. ferina, L.	European pochard	Less common
Glaucionetta clangula clangula (L.)	European goldeneye	Less common
Cygnus olor (Gmelin)	Mute swan	Common
Charadriiformes Scolopacidae		
Capella gallinago gallinago (L.)	Snipe	Less common
Laridae		
Larus ridibundus ridibundus, L.	Black-headed gull	Common
Gruiformes Rallidae		
Gallinula chloropus chloropus (L.)	Moorhen or waterhen	Very common
Fulica atra atra, L.	Coot	Very common
Coraciformes Alcedinidae		
Alcedo atthis inspida, L.	Kingfisher	One or possibly 2 pairs
Passeriformes Motacillidae		
Motacilla alba yarrelii, Gould.	Pied Wagtail	Common

In addition the song thrush (*Turdus ericetorum philomelus*) occurs very commonly in the nearby wood and feeds often on water-snails.

A variety of vertebrates was used as experimental definitive hosts, namely, ducks, chickens, pigeons, mice and canaries, and these were maintained in the departmental animal houses. Day-old ducks and chicks were obtained from dealers, but some of the pigeons and all of the mice were bred in the laboratory. All the birds were fed on dry commercially supplied special food preparations, which were assumed to be free from larval trematodes. As a precaution, samples

of the faeces of all experimental definitive hosts were examined daily for trematode eggs for one week before the commencement of any experiment.

In all, over 4000 specimens of *Lymnaea stagnalis* were examined during the 4-year period 1955–59. Emergent cercariae of *Cotylurus brevis* were found only in summer and autumn, and especially in June, July and August. During these 3 months, about 25% of the snails examined were found to emit cercariae. In winter months, dissection of specimens of *L. stagnalis* revealed that about 25% harboured sporocysts containing immature cercariae of *C. brevis*, but that nearly all specimens were acting as second intermediate hosts to *C. brevis*, i.e. they contained tetracotyle larvae. From time to time other pond snails (listed on p. 552) were examined, but on only one occasion were cercariae of *C. brevis* present: one specimen out of over 100 *L. pereger* examined was infected with cercariae of *C. brevis*.

Trematode cysts were introduced orally into experimental definitive hosts with an ordinary medicinal pipette. Following the introduction of such cysts, samples of the host faeces were sedimented and examined daily with a stereomicroscope for trematode eggs. Adult trematodes were recovered by killing the infected host with chloroform, and then transferring the gut into a suitable saline; the contents of the gut were searched with a stereomicroscope.

STAGES IN THE LIFE HISTORY

Egg capsules (Fig. 1)

Egg capsules collected from freshly passed faeces of pigeons measure 0·096 × 0·064 mm. (0·088–0·104 × 0·057–0·068 mm.) when examined without the pressure of a cover-glass. They are yellowish brown, operculate, and thin-shelled, and are easily broken by the pressure of a cover-glass or when being pipetted.

In capsules collected from freshly deposited faeces, the zygote nucleus has already divided into two, but the cytoplasm remains undivided. In this respect *Cotylurus brevis* resembles two other strigeids *Alaria arisaemoides* and *A. canis* as described by Pearson (1956). In a sample of thirty-five capsules, the zygote and its derivatives were near the operculate pole in thirty-two, and near the aboperculate pole in the remaining three.

Miracidium

Repeated attempts were made to hatch miracidia from egg capsules, but on only two occasions were they successful. The greatest difficulty encountered was in obtaining supplies of eggs. Even though on one occasion twenty-six worms were recovered from the intestine of a pigeon, the number of eggs collected from the faeces deposited during the previous 18 hr. was only three. The maximum number of eggs collected from a single sample of faeces weighing about 25 g. was seventy-three, but most of these eggs did not develop. Most of the observations, therefore, were made upon embryos still contained within capsules.

Temperature variations between 19° and 24° C. appeared to have little or no effect on the rate of embryonic development, the total time being always about 3 weeks, which is very similar to that known for some other strigeids, for example,

Strigea tarda, SteenstR. as given by Mathias (1925) and *Diplostomum flexicaudatum*, Cort & Brooks as described by van Haitsma (1931*b*). The embryo lies nearly always with its anterior end directed towards the operculate pole. Hatching was not observed.

The shape of the miracidium is typical of that of other digenetic trematodes, and the embryo measures 0·1–0·154 × 0·055–0·072 mm. The extreme anterior end of the miracidium, the apical papilla, is blunt and retractile. The body, excepting the apical papilla, is covered with cilia that may occasionally be seen to beat. There are two dark-brown to black eye-spots, each measuring 9 × 4μ, separated

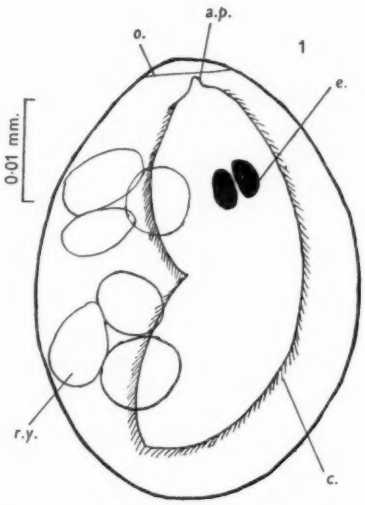


Fig. 1. Capsule of *C. brevis* with embryo.

from each other by a distance of about 3μ. In some miracidia the eye-spots are contiguous, for example, *Echinoparyphium flexum*, Linton (Najarian, 1954), and in others relatively widely separated, for example, *Strigea tarda*, SteenstR. (see Mathias, 1925), and *Diplostomum flexicaudum*, Cort & Brooks (see van Haitsma, 1931*b*).

I was unable to observe further details of the anatomy of the miracidium of *Cotylurus brevis*, but have no reason to suppose that the structure would be different from that of the miracidia of *Strigea tarda* and *Diplostomum flexicaudum*.

Most of the eggs which contained fully differentiated miracidia were used for attempts to infect laboratory-bred *Lymnaea stagnalis*, but all my attempts were unsuccessful. The interval between the penetration of the miracidium into the mollusc and the emergence of the cercariae in some species related to *Cotylurus brevis* is, in *Strigea tarda*, 6–7 weeks according to Mathias (1925), in *Cotylurus communis*, 40 days, according to van Haitsma (1930), in *C. flabelliformis*, 6 weeks, according to van Haitsma (1931*a*), in *Diplostomum flexicaudum*, 45 days, according to van Haitsma (1931*b*), and in *Fibricola cratera*, 25–28 days, according to Cuckler

(1949). It seems likely then that a period of about 6 weeks intervenes between the infection of the snails by the miracidia and the emergence of the cercariae in *Cotylurus brevis*.

Sporocyst

The cercariae of *C. brevis* develop in unbranched sporocysts, an adequate description of which has been given by Dubois (1934).

Cercaria (Fig. 2)

A detailed morphological study of the cercaria of *Cotylurus brevis* has shown that it is so similar to '*Cercaria helvetica XXXIV*' described by Dubois (1934) from the same host species *Lymnaea stagnalis* from Lake Neuchâtel, Switzerland, that a complete redescription would be superfluous. Instead I include (Table 2) a comparison of the measurements of specimens of the cercaria of *Cotylurus brevis* from Edgbaston Pool with those given by Dubois for *Cercaria helvetica XXXIV*, and add comments, below, about certain morphological features.

Table 2.

(Measurements (mm.), based on living material.)

	Cercaria of *C. brevis* (present investigation)	*C. hevetica XXXIV* (Dubois, 1934)
Body		
Length	0·156–0·300	0·120–0·360
Width	0·060–0·112	0·030–0·110
Tail-stem		
Length	0·160–0·280	0·200–0·240
Width	0·036–0·060	0·036
Furcae, length	0·180–0·300	0·240–0·270
Anterior organ		
Length	0·032–0·060	0·050
Width	0·032–0·044	0·036
Acetabulum, diam.	0·039–0·046	0·029–0·036
Pharynx, diam.	0·011–0·014	0·012–0·013
Oesophagus, length	0·019–0·025	0·010–0·030

(1) In his specific diagnosis Dubois states that the ventral sucker has three concentric rings of spines. In general my specimens agree with this description, but sometimes the spines are somewhat irregularly arranged. In some specimens, moreover, at least in a part of the ventral sucker, four and sometimes even five, rows of spines are present. Recognition of the occurrence of this intra-specific variation in *Cotylurus brevis* is important because the number of rings of spines on the ventral sucker has been used (Dubois, 1934) to distinguish *Cercaria helvetica XXXIV* with three rows from *C. douglasi*, Cort & Brooks, as redescribed by Olivier & Cort (1941) with several irregular rows of thin, elongate spines.

(2) Similarly the twenty or so spines on the dorsal lip of the mouth may be more irregularly arranged than is suggested by Dubois's description 'disposés sur 2 rangées alternates'.

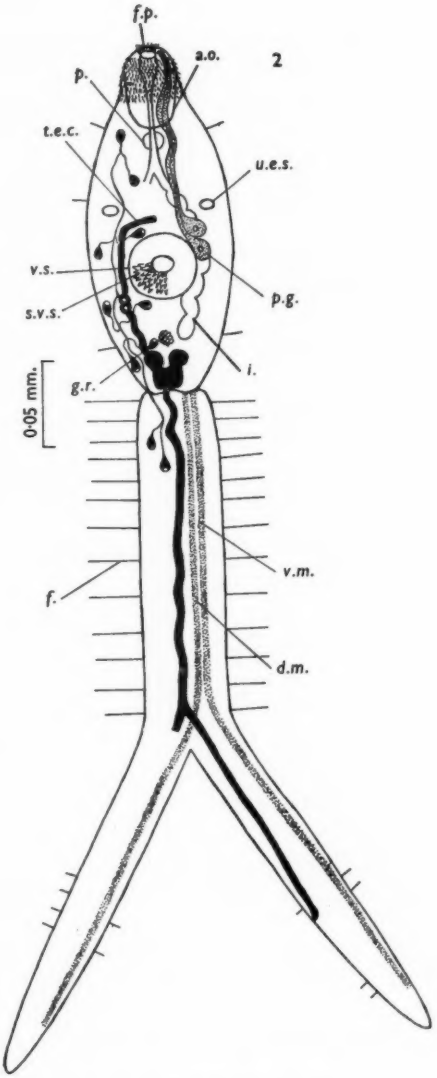


Fig. 2. Cercaria of *C. brevis*.

(3) The number of rows of spines behind the mouth region (see Dubois, 1934, fig. 2) has been found to vary between eleven and fourteen.

(4) The oesophagus of the cercaria of *Cotylurus brevis* is 'short' in comparison

with the 'long oesophagus' of some other strigeid cercariae, for example '*Cercaria strigea tardae*' as described by Mathias (1925) and *Cercaria A*, Szidat as described by Szidat (1924), by Dubois (1929), and by Wesenberg-Lund (1934), but not quite as short as in *C. helvetica XXXIV* as described by Dubois. Dubois stated that the length of the oesophagus in *C. helvetica XXXIV* varied between 16 and 26 % of the distance between the posterior border of the pharynx and the anterior border of the ventral sucker; the corresponding values in a sample of ten living cercariae of *Cotylurus brevis* were 28–40 % (mean 34 %).

(5) The musculature of the tail, best seen in polarized light, consists of four simple, unbranched bundles running the whole length of the tail-stem and passing to the furcae, each of which receives two of these bundles, one to its dorsal edge and the other to its ventral edge.

(6) Anterolateral to the acetabulum on the dorsal surface of the cercaria of *C. brevis* is a pair of unpigmented eye spots that were not referred to in Dubois's account of *Cercaria helvetica XXXIV*. The term 'unpigmented eye-spots' was used by Cort & Brooks (1928) for corresponding structures in their '*Cercaria douglasi*', but similar bodies in *C. strigea tardae* were regarded by Wesenberg-Lund (1934) as 'glomeruli connected with each other by a transversal commissure'. In my specimens of the cercaria of *Cotylurus brevis* there appeared to be no connexion between the 'unpigmented eye-spots' and the excretory system.

Emergence and behaviour of cercariae

When freshly collected snails were brought into the laboratory, the cercariae of *C. brevis* were usually already emerging from the infected snails. This form of emergence thus differed from that in snails infected with stylet or echinostome cercariae, where generally no emergence took place during the first hour in the laboratory, and often emergence did not begin until after 2 days.

Sometimes snails infected with cercariae of *C. brevis* ceased to emit cercariae, the emission being subsequently resumed. One snail discharged cercariae continuously over a period of 118 days; then there was a cessation of emission for 21 days, followed by a period of renewed emission. In another snail dissected during a period of non-emergence it was found that none of the thirty-three sporocysts present had any fully developed cercariae; only germ balls and early developmental stages of cercariae were present.

It appeared that a daily rhythm in the emission of cercariae was superimposed on the long periods of active emission. Hourly observations on the infected snails in May 1956 showed that there was practically no emergence before 10 a.m., but that after that there was for a period a very high rate of emergence (several hundreds per hour), which continued until mid-afternoon, when the emergence appeared to cease abruptly, not to be resumed until the following morning.

This pattern of the emergence of the cercariae of *C. brevis* during the day time contrasts markedly with that known for some other cercariae, for example, the cercaria of *Plagiorchis* (*M.*) *megalorchis* described by Rees (1952), which has also been found in Edgbaston Pool. The emergence of this cercaria is confined to evening,

night and early morning hours. The reason for this difference in emergence between the cercaria of *P.* (*M.*) *megalorchis* and that of *Cotylurus brevis* is obscure, but it is obvious that the general statement by Dubois (1929) that the emergence of cercariae is influenced mainly by the effect of temperature on the metabolism of larval trematodes needs further investigation.

On escape from their snail host the cercariae of *C. brevis* immediately dart in all directions and become uniformly distributed in the water. They do not exhibit any consistent behaviour in response to light or shade. Freshly emerged cercariae continue to swim, apparently aimlessly, with an occasional pause of a second or so. The cercaria usually swims, tail foremost, by means of side to side lashing of the tail, but very occasionally it spins round and round and descends.

Sometimes the cercaria becomes attached temporarily, by means of its acetabulum, to any convenient substratum. When it is thus attached the body lies usually in a horizontal plane, while the tail is held roughly vertically. When it is in this position, the cercaria may move along in a leech-like manner; usually the tail-stem and furcae remain still during this form of locomotion, but sometimes they may vibrate as in swimming. While still in this resting position on the surface of an object, the body may not creep along, but the cercaria may rotate on its ventral sucker, with the tail-stem and the furcae either moving or at rest.

After a free-swimming period of 6–8 hr., the periods of inactive suspension in water become extended to about 45 sec. and eventually the cercariae appear to become exhausted and settle on the bottom. A symptom of the approaching death of a cercaria is a characteristic curling of the furcal rami.

Penetration of the cercariae into secondary intermediate hosts

In the life cycle of the cercaria of *C. brevis* the same species of snail, *Lymnaea stagnalis*, serves as both the primary and the secondary intermediate host; even the same individual snail may serve in both capacities. Three laboratory-bred specimens of *L. auricularia* and two of *L. pereger* were successfully infected with cercariae of *Cotylurus brevis* but all five snails died before the tetracotyle stage of the infection was reached. *Planorbis corneus* and *P. carinatus* were found to be resistant to penetration by the cercariae of *Cotylurus brevis*.

When a snail which is discharging cercariae of *C. brevis* is kept with other (uninfected) laboratory-bred specimens of *Lymnaea stagnalis*, the cercariae do not seem to become aware of the presence of these potential hosts, but keep on swimming aimlessly. If by chance they make contact with the exposed part of a snail, penetration is usually effected.

In the course of penetration the cercaria takes a firm grip with its ventral sucker while the anterior end of the body, intermittently, explores the area around. The tail-stem is usually held at right angles to the body with the furcae stretched apart; the tail-stem is then suddenly cast off with an abrupt convulsion. Less frequently the cercaria fixes itself with its anterior organ only, the ventral sucker not being employed; the whole of the body is then in line with the tail-stem with the furcae stretched apart, as when the cercaria is suspended in water. I failed to observe

the complete process of penetration by the cercaria of *Cotylurus brevis*, but Mathias (1925) and Szidat (1924) have described a period of up to 30 min. for the penetration of the cercariae of *C. cornutus* into *Lymnaea stagnalis*.

Within 24 hr. of the penetration into the second intermediate host by cercariae of *Cotylurus brevis*, the larvae have all reached the digestive gland.

Developmental stages in the second intermediate host (Figs. 3–8)

As in the the only other Cotylurinid life cycle known, namely, that of *C. cornutus*, Rud., as described by Szidat (1924, 1929), the development of *C. brevis* in its second intermediate host, *Lymnaea stagnalis*, involves a holometabolic metamorphosis. During this process most of the cercarial organ-systems are completely broken down; in fact, only the excretory system and the genital rudiments persist unaltered. Certain features which are peculiar to the developmental stages in the second intermediate host have only a temporary existence, and finally there is the gradual appearance of the characters of the next larval stage, namely the encysted tetracotyle.

Observations on the development of *Cotylurus brevis* in its second intermediate host (hereafter referred to as 'precystic devēlopment') were made during the summers of 1956 and 1957 and were repeated in the autumn of 1958. In the summer observations, precystic development was completed by the envelopment of the encysted tetracotyle in a gelatinous sheath, in 23 days, but in the observations of autumn 1958, although the laboratory temperature was the same (19°–24° C.), development was slower, for, after 40–45 days the stage reached corresponded only with that reached after 15–20 days in the 1956 nd 1957 observations. An account based on the 1956 and 1957 observations will now be given of the stages of the precystic development of *C. brevis*.

Size

During the first 8 days there is no significant change in the size of the precystic stages, the body measuring 0·136–0·280 × 0·064–0·104 mm., but after the eighth day there is a gradual increase in size, until on the fifteenth day the maximum size of 0·640–0·972 × 0·334–0·592 mm. is attained. Following this period of growth there is a gradual decrease in size, and on the twenty-third day, i.e. when the fully formed tetracotyle stage is reached, the larva has returned to about the size of the body of the free-swimming cercaria (0·200–0·264 mm. long).

Loss of cercarial organs

During the first 24 hr. after entry into the second intermediate host, the penetration glands and their ducts begin to degenerate and are almost completely lost by the end of the third day. By the fourth day the cuticular spines are smaller and the eye spots can no longer be seen. By the sixth day the cuticular spines are further reduced in size and persist only in the anterior region of the body and by the eighth day they have disappeared. During the next 5 days, i.e. from the ninth

to the thirteenth day inclusive, the digestive tract disintegrates, the parts disappearing in posterior to anterior succession. Of the cercarial organs that are destined to disappear, the acetabulum is the last to disintegrate.

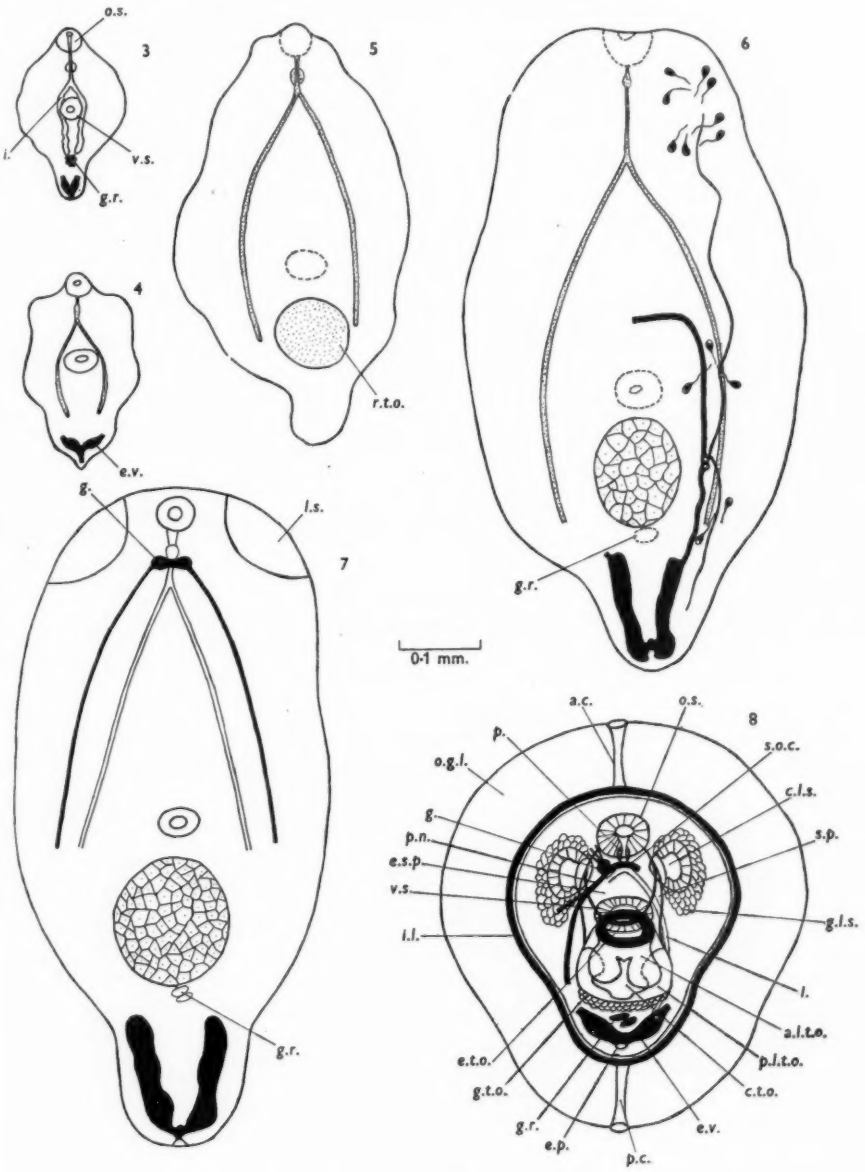


Fig. 3–8. Stages in the holometabolic metamorphosis of *C. brevis*.

Persistent cercarial organs

During the whole process of metamorphosis, the excretory system persists, although after the eighth day the flickerings of the flame cells appear to be more sluggish and the excretory tubules are considerably less evident. By the thirteenth day, however, when other organs are still degenerating, the excretory system has become further elaborated: the two arms of the bladder are greatly distended, and the main collecting tube of each side arises, not terminally as in the cercaria, but from the antero-lateral region of the corresponding arm of the bladder; the transverse anterior excretory commissure is unchanged, and the anterior and posterior collecting tubes are very prominent (Fig. 6). The number of flame cells in the body of the 13-day-old pre-cystic stage has increased from sixteen to twenty-six. The number and grouping of flame cells suggests that each flame cell of the pre-acetabular region in the cercaria has divided into two.

My observations on the excretory system therefore differ from those of Dubois (1934), who found precystic stages of *Cercaria helvetica XXXIV* (= the cercaria of *Cotylurus brevis*), in *Lymnaea stagnalis* and remarked that in larvae 1·00–1·20 mm. long (i.e. about the same length as my 13-day precystic stage), while the excretory tubes on each side of the body remained distinct, the flame cells were no longer apparent.

After the thirteen day the number of flame cells continues to increase and in precystic stages of between 15 and 21 days I have counted over 100 flame cells.

Characters peculiar to the precystic stages

On and after the eighth day the body becomes more and more flattened and transparent and the development of large vacuoles gives it a typical reticulate appearance. Here and there in the body are scattered large, pyriform cells 'the Riesenzellen' of Szidat (1924) or 'cellules géantes' of Dubois (1934), which measure about 0·013 mm. in diameter. With the movements of the body these cells may become localized in various places, for example, there may be a temporary concentration at one or other side of the body. Each of these 'giant cells' bears a fine thread-like prolongation at the narrow end. On the thirteenth day the 'giant' cells have decreased in size and measure 0·006 mm. in diameter, but their protoplasmic prolongations are clearly linked with each other. On the fifteenth day there are present in the body certain globular bodies of variable size in which no nucleus could be seen and some of them contained a few refractile bodies similar to those present in the 'reserve bladder' of the tetracotyle; they do not stain with Sudan IV and do not exhibit any particular pattern of distribution; these globular bodies have a tendency to aggregate into clumps which are moved about with the movements of the body.

Precystic developmental features of the tetracotyle stage

On the twelfth day, while the general disorganization of various cercarial organs is still going on, the rudiments of the tribocytic organ make their appearance as

a conspicuous spheroidal mass of finely granular material behind the acetabulum (Fig. 5).

The tribocytic organ (a term proposed by Baer, 1932) in the genus *Cotylurus*, as described by various workers, for example, Mathias (1925), van Haitsma (1931*a*) and La Rue (1932), is a bilobed structure located posterior to the ventral sucker, and various functions have been attributed to it. Brandes (1890), Muhling (1898), Szidat (1929), La Rue (1927, 1932) and Haitsma (1931*a*) were of the opinion that its secretion is histolytic, but Krause (1914) believed that this secretion has no corrosive action.

On about the fifteenth day the vacuoles that are so prominent in previous stages appear to become smaller and to contain more granules, and the body is of a generally firmer consistency. The body has now become differentiated into a smaller, narrower hind-body, and a larger, broader fore-body. The oral and the ventral suckers begin to appear (Fig. 5, 6). There is, however, an interval from the thirteenth to the fifteenth day when no acetabulum is recognizable. The digestive tract is in the course of reorganization. Overlying the posterior part of the pharynx is a transverse nerve commissure, uniting the two ganglionic masses lying one on each side of the pharynx (Fig. 7). From this 'cerebral region' there runs posteriorly on either side, externally to the intestine, a thick nervous strand. The primordium of the tribocytic organ may now be seen to be cellular, and it has become more compact, and posterior to it are the genital rudiments (Figs. 6, 7). The excretory tubules and the flame cells, whose number has probably increased, are not easily seen, because the body is becoming more and more opaque. The excretory pore is dorsal in position. The transparency and the reticulations of the body are reduced, but four or five large reticular spaces remain around the ventral sucker.

Between 15 and 21 days after the infection of the snails, the precystic stages become transformed, gradually, into the typical tetracotyle shape. This tetracotyle stage is capable of contraction and extension but does not move from place to place. It measures 0·44–0·568 × 0·416–0·584 mm. By the twenty-first day both suckers are completely organized. The ventral surface in the region anterior to the tribocytic organ is hollowed out so as to assume the shape of a basin, on each side of which, posterior to the oral sucker, is a lateral sucking cup which is considerably larger than the oral sucker. The ventral sucker lies in the depth of the cup-shaped fore-body and is overlapped by the anterior lip of the tribocytic organ. From time to time the fore-body may lose, temporarily, its concavity and when this happens the lateral sucking cups may become directed anteriorly. The cavities of the lateral sucking cups become more pronounced and communicate with the general concavity of the fore-body. The wall of each lateral sucking cup is composed mostly of radially running fibres. The tribocytic organ is now differentiated into a pair of lips lying one in front of the other, and the alimentary canal is completely organized into an oral sucker, pharynx, oesophagus, and the intestinal caeca.

The body has become more and more opaque, but it is still possible to determine that the reticulations of the body have disappeared. The refractile granules referred

to previously are now aggregated into clumps which will eventually form the 'reserve bladder' system of the tetracotyle described below. The same kind of granules are also present in the excretory vesicle but they are not yet forced out through the excretory pore as in the fully formed tetracotyle.

Tetracotyle (Fig. 8)

The size of the fully formed encysted tetracotyle (i.e. on the twenty-third day after the penetration of the cercaria) including the cyst wall but excluding the gelatinous covering which usually overlies the early formed tetracotyle, and measured without pressure of a cover-glass, is 0·446–0·54 × 0·40–0·50 mm. Subsequently there is often a considerable decrease in the size of the cyst, but the decrease does not always proceed to the same limit, and the shrunken cysts may be arranged according to size into two groups: 'large cysts' (0·360–0·40 × 0·290–0·310 mm.), and 'small cysts' (0·204–0·269 × 0·216–0·257 mm.). Usually (in about three snails out of four) the cysts after their 'shrinkage' are all 'large cysts', but sometimes (in about one snail out of four) 'large cysts' and 'small cysts' are present in equal proportions.

It was later found that there was no morphological difference between adults bred out in pigeons from cysts of three different sizes, i.e. (*a*) before shrinkage, (*b*) shrunken 'large cysts' and shrunken 'small cysts'.

For the purpose of comparison with the tetracotyle stages of other species the following measurements, taken from a random sample of thirty-five cysts taken from all three size-groups referred to above and fixed in Gilson's fluid, are as follows.

Fore-body: length, 0·168 (0·144–0·184) mm.; breadth, 0·244 (0·208–0·232) mm.

Hind-body: length, 0·072 (0·056–0·080) mm.; breadth, 0·120 (0·112–0·132) mm.

The fully developed tetracotyle is pyriform, being invested by a cyst wall composed of two layers, an outer 'gelatinous' layer 0·039–0·048 mm. thick and a tough opaque inner layer, 0·003–0·004 mm. thick. The gelatinous layer is composed of a non-granular material and in older cysts this layer can no longer be seen, but eventually, in tetracotyle known to be a year old, the gelatinous covering has disappeared altogether; the exact period of persistence of the gelatinous covering is not known. When treated with 70 % alcohol, after fixation, the gelatinous covering shrinks and ultimately, in absolute alcohol, it is lost altogether. This accounts for the fact that, whereas in sectioned material the tough inner cyst wall persists, the gelatinous layer is invariably wanting. In living material the gelatinous layer may easily be removed with needles, but the inner layer is much tougher. Occasionally dead and decaying tetracotyles were found still enclosed in their gelatinous capsules.

According to Pearson (1959) the gelatinous covering of the tetracotyle of *Strigea elegans* is divisible into two parts 'of about equal thickness, an outer non-granular matrix containing scattered clumps of corpuscles and granules and an inner half with a somewhat friable granular matrix'. In the tetracotyle of *Cotylurus brevis* the gelatinous layer is non-cellular and of a homogeneous nature, with no differentiation into sublayers. Spermatozoa from the host snail, debris and the refractile

granules forced out through the excretory pore of the parasite often become entangled in this layer and this could lead to erroneous interpretations.

Various other species of tetracotyle: for example *Tetracotyle cornuta*, *T. communis*, *T. diminuta*, *T. intermedia* and *T. bonasae*, have been described by Szidat (1924, 1929), Hughes (1928*c*) and Chandler (1954) to be enclosed in gelatinous coverings. Hughes (1929) in his description of *T. flabelliformis*, Faust, recognized only one layer: 'a thick layer of gelatinous substance closely adherent to the cuticle of the parasite, with perforations adjacent to the excretory pore, and the external openings of the oral sucker, lateral suckers and the cavity of the holdfast organ'; this layer, apparently corresponds to the gelatinous covering of the tetracotyle of *Cotylurus brevis*.

Since it has been found, as is described above, that the gelatinous covering of the tetracotyle of *C. brevis* disappears it is possible that a similar covering, as yet unobserved, is also present in other tetracotyle larvae, for example, *Tetracotyle orientalis*, Faust and *T. serpentis*, Hughes.

The gelatinous covering is perforated at each end of the body by a canal which is dilated distally (Fig. 8). It is difficult to observe this canal at the anterior end of the parasite but at the posterior end it is very prominent. The posterior canal is in direct communication with the excretory pore of the larva and frequently, when cysts are lying in dishes for microscopical examination, the refractile granules of the reserve-bladder system may be seen to be forced out through its aperture. Szidat (1924, 1929), Harper (1931) and Wesenberg-Lund (1934) have shown the presence of such canals in *T. cornuta*, while Pearson (1959) could find 'no passages through the cyst wall' of the tetracotyle of *Strigea elegans*.

The tough inner layer is perforated in places corresponding to the apertures of the oral and the ventral suckers, lateral sucking cups, tribocytic organ and the excretory pore.

In the median region the ventral body wall is concave and the margins of the concavity are distensible. This concavity has been designated by Faust (1918) as the 'suctorial cup or pocket' and this term was later adopted by Hughes (1928) In the freshly formed tetracotyle, the suckers, lateral sucking cups and tribocytic organ all open into the suctorial pocket, but in older specimens the whole of the oral sucker is anterior to the anterior border of the suctorial pocket and the lateral sucking cups open partly inside and partly outside the concavity.

The oral sucker is subterminal, with its aperture directed anteriorly. In living specimens its diameter ranged from 0·046 to 0·061 mm., and in balsam preparations from 0·041 to 0·048 mm. Pearson (1959) has described the presence of certain glands in the oral sucker of the tetracotyle of *S. elegans*, but no such glands appeared to be present in the tetracotyle of *Cotylurus brevis*; Pearson also suggests that 'these glands may be cystogenous and secrete the hyaline, tough material of the parasite cyst' as 'the parasite cyst is appreciably thicker over the anterior end of the tetracotyle than anywhere else'. Oral glands have not been described in any other species of tetracotyle.

The ventral sucker is situated mostly in the posterior region of the suctorial pocket, and is often obscured by the protruding lips of the tribocytic organ. When

the opening of the suctorial pocket contracts, its margins may completely hide the ventral sucker. The ventral sucker lies much more deeply in the body parenchyma than does the oral sucker. This is in direct contrast with the observations of Pearson (1959), on *Strigea elegans*, in which the ventral sucker is capable of protrusion on a 'bulbous extension of the fore-body wall'. The tetracotyle of *S. elegans* appears to be unique in this respect. In living material the ventral sucker is transversely oval, but in fixed preparations it is usually isodiametric (0·046–0·055 mm).

In the antero-lateral region of the fore-body, on either side of the suctorial pocket, is a compact mass of tissue enclosing a cavity, nearly equal in size to the oral sucker, and opening through an oval aperture directed obliquely towards the acetabular region. These structures have been earlier named 'lateral sucking cups' (Hughes, 1928), 'accessory suctorial grooves' (Faust, 1918), 'lateral pseudosuckers' (Mathias, 1925), 'lateral suckers' (Tubangui, 1933) and 'pseudosuckers' (Pearson, 1959). These lateral sucking cups are glandulo-muscular organs and are capable of great distension. The thick walls of these cups, on all but the mesial side (which is occupied by the aperture) are traversed by radiating muscle fibres. In late precystic stages delicate extrinsic muscle strands may be seen to radiate from the sides of these cups to the dorsal walls of the fore-body. The cups are surrounded almost completely by unicellular glands (stained with light green in Masson's trichrome technique) with an especial concentration of them posterolaterally to each sucking cup. The apertures of these cups measure 0·048–0·059 × 0·021–0·026 mm., and may be stretched to nearly twice this size. About 5 days before the development of the tetracotyle is completed, the lateral sucking cups are eversible, but in the fully formed tetracotyle I have never seen them to be evaginated.

The lateral sucking cups persist apparently without morphological change into the adult *Cotylurus brevis*, where I have observed them to function as adhesive organs, their cavities being filled with intrusions of host tissue. The nature of the lateral sucking cups in the tetracotyle stage of other species has from time to time been the subject of conflicting reports, for example, Faust (1918, 1922), Mathias (1925) Hughes (1928) and Pearson (1959), have variously described the lateral sucking cups to be non-muscular, muscular, glandular, and glandulo-muscular organs.

The well-developed tribocytic organ is situated mainly in the fore-body, immediately behind the ventral sucker, its average size being 0·121 × 0·187 mm. in living specimens and 0·083 × 0·107 mm. in fixed material; in some permanent preparations it is nearly isodiametric with a diameter of about 0·090 mm. It consists of an anterior and a posterior lip, projecting from the dorsal and posterior walls of the suctorial pocket, and enclosed in a cavity which opens into the inside of the suctorial pocket through a transversely elongated aperture; the latter measures 0·052–0·083 mm. in transverse diameter in life. The anterior border of the opening frequently overlies the posterior region of the acetabulum. The anterior lip is deeply cleft, so that it forms two definite halves, but the posterior lip is only slightly notched and shows considerable variations in shape and size (Fig. 8). Both lips

are often extruded so that they cover the acetabulum completely; alternatively they may be withdrawn and assume a very folded appearance, when they may be readily confused with other organs.

The gland of the tribocytic organ is a compact, multinucleate, disk-shaped structure, situated immediately behind the posterior border of the tribocytic organ. This gland is strongly positive to the periodic acid-Schiff test for polysaccharides. No definite ducts or openings associated with the gland were observed either in living or sectioned material. However, Pearson (1959) has figured the secretion of the tribocytic gland, in the tetracotyle of *Strigea elegans*, (Chandler and Rausch), immediately behind the gland itself.

The digestive system as a whole, especially the intestinal caeca posterior to the acetabulum, is exceedingly difficult to observe in living material. The prepharynx is very short; it is followed by a small muscular pharynx, which measures, in living material, about 0·025 mm in diameter. Sometimes the pharynx overlies the posterior region of the oral sucker. The oesophagus is about the same length as the pharynx. The whole of the digestive tract may be stained faintly with neutral red. The intestinal caeca, immediately after the oesophageal bifurcation, are slightly narrower than the oesophagus itself, but gradually increase in diameter posteriorly until they terminate dorsally to the lobes of the excretory vesicle.

The nervous system consists of two ganglionic masses, one on either side of the oesophagus immediately behind the pharynx and united by a thick supraoesophageal commissure. Each of the ganglia gives off three nerve strands, two anteriorly and one posteriorly.

Two transversely elongated and deeply staining masses, lying posterior to the gland of the tribocytic organ and encompassed by the posterior ends of the intestinal caeca, are presumably the rudiments of the genitalia of the adult.

The excretory system is divisible into two portions, which in other tetracotyle larvae have been described (Faust, 1922; Hughes, 1927, 1928*a*, 1928*b*, 1928*c* and 1929) as the primary excretory apparatus consisting of flame cells with their associated capillaries, and the reserve bladder with calcareous granular inclusions.

The reserve bladder of the tetracotyle of *Cotylurus brevis* is similar to that described by Hughes in other tetracotyle larvae as 'an irregular, continuous, coarse-meshed network of spaces filled with a hyaline fluid containing calcareous granules of various sizes, which move freely in any direction in response to pressure or movements of the body' and thus differs fron the 'regular system of wide canals' described by Faust (1922) in *Tetracotyle orientalis* and *T. flabelliformis*.

The excretory vesicle occupies most of the space in the hind-body and is divided into two lobes. The excretory pore, which is about 0·014 mm. in transverse diameter, is ventral in position, at a distance varying between 0·016 and 0·039 mm. from the extreme posterior end of the body. The refractile granules of the reserve bladder may be forced out through the excretory pore in large puffs, especially when the body is compressed.

The complete excretory system of the other species of tetracotyle, including the primary excretory apparatus and the reserve bladder system, has been described in great detail by Faust and Hughes.

Degree of infestation of snails with tetracotyle larvae

In a sample of 100 specimens of *Lymnaea stagnalis* of various sizes collected from Edgbaston Pool, 98 were infected with post-cercarial stages of *Cotylurus brevis*. The number of tetracotyle per snail varied considerably: 65% of the snails harboured several hundreds (sometimes over 3000) precystic or tetracotyle stages, 25% harboured from 100 to 200 and 10% less than 100. Under laboratory conditions cercariae of *C. brevis* will continue to penetrate snails almost indefinitely. The snail does not appear to develop any immunity to further penetration.

Comparison with related species

The tetracotyle of *C. brevis* resembles most closely the tetracotyle stages of two other species of the genus *Cotylurus: C. flabelliformis*, Faust (1917), as described by Hughes (1929) and by van Haitsma (1931*a*), and *C. cornutus* (Rud.) Szidat (1928), as described by Hughes under the mame of *Tetracotyle cornuta* and by Mathias (1925), Szidat (1924) and Harper (1931).

These three tetracotyles are almost identical with each other morphologically, the only difference being size. In the present investigation, however, and in the case of *T. pipientis* (Faust), Hughes (1938), it has been shown that size variation in tetracotyle is not of taxonomic significance, and it is concluded, therefore, that the tetracotyle of *Cotylurus brevis* may be distinguished from that of *C. flabelliformis* and *C. cornutus* only by a knowledge of other stages in the life cycle.

Summary of experimental infection of definitive hosts

Mice, ducks, chicks and pigeons were employed as experimental definitive hosts, but successful results were obtained only with pigeons. Except where otherwise stated, the tetracotyle cysts used in these experiments were obtained from experimentally infected, laboratory-bred snails. An outline of the various experiments is given below.

Experiment 1. Pigeon 1, fed with 1230 tetracotyle cysts on 1. i. 57; faeces examined on alternate days; dissected on 8. i. 57, negative.

Experiment 2. Pigeon 2, fed with 1200 cysts on 23. i. 57; faeces positive on 29. i. 57 (twenty eggs); dissected 29. i. 57, two adults from small intestine.

Experiment 3. Pigeon 3, fed with 400 cysts on 31. i. 57; three eggs from overnight faeces on 6. ii. 57; reinfected with 1600 cysts on 8. ii. 57, and 1100 on 9. ii. 57; faeces positive every day from 6. ii. 57 to 14. ii. 57; dissected on 14. ii. 57, twenty-six adults in groups of seven and six in duodenum, thirteen others scattered along intestine more posteriorly.

Experiment 4. Pigeon 4, fed with 150, 500 and 240 cysts on 31. i. 57, 1. ii. 57 and 4. ii. 57, respectively; nine eggs from overnight faeces on 6. ii. 57; dissected on the same date, two adults from small intestine.

Experiment 5. Pigeon 5, fed with 1640, 1837, 987 and 2100 cysts on 11. ii. 57, 13. ii. 57, 14. ii. 57 and 12. ii. 57, respectively; fifty-nine eggs from overnight faeces on 18. ii. 57 and two eggs on 19. ii. 57; eggs disappeared from faeces on 20. ii. 57; dissected on the same date, negative.

Experiment 6. Pigeon 6 (laboratory-bred) fed with 343 cysts (44 days old) on 22. ii. 57; fifteen eggs from faeces on 28. ii. 57, seventy-three eggs on 1. iii. 57 and eight on 6. iii. 57; faeces negative on 11. iii. 57; dissected on the same date, negative.

Experiment 7. Pigeon 7, fed with over 2000 naturally developed cysts on 18. iii. 57; five eggs from overnight faeces on 24. iii. 57; no eggs on 25. iii. 57, but one partially decomposed adult recovered from faeces on same date.

Experiment 8. Pigeon 8, fed with thousands of cysts from naturally infected snails on 18. iii. 57; 378 on 20. iii. 57, 121 on 21. iii. 57, 1500 on 27. iii. 57 and twenty-five on 28. iii. 57; faeces positive on 24. iii. 57, 25. iii. 57, 26. iii. 57, 27. iii. 57, 29. iii. 57 till 1. iv. 57; dissected on 2. iv. 57, nine adults, in groups of five and four in small intestine.

Experiment 9. Pigeon 9, 565 cysts from natural infections on 27. iii. 57; dissected on 2. iv. 57, negative.

Experiment 10. Pigeon 10 (laboratory-bred) fed with 127 cysts from natural infections on 1. iv. 57, forty-three on 15. iv. 57, over 2000 on 25. iv. 57; faeces positive from 7. iv. 57 to 13. iv. 57 and negative from 14. iv. 57 to 20. iv. 57; positive again from 21. iv. 57 to 23. iv. 57, becoming negative again from 24. iv. 57 to 2. v. 57; dissected on the same date, negative.

Experiment 11. Pigeon 11 (laboratory-bred), fed with thousands of cysts on 3. v. 57; eleven eggs from overnight faeces on 8. v. 57, and seventeen on 9. v. 57; dissected on 9. v. 57, three adults at about 14 cm. behind the duodenal loop, and two more individuals at about 3 cm. behind the first group.

Experiment 12. Pigeon 12, fed with 1700 cysts (23 days old) on 13. vi. 57; faeces negative till 20. vi. 57; dissected on the same date, negative.

Experiment 13. Pigeon 13, fed with 476 cysts (23 days old) on 13. vi. 57; faeces negative till 22. vi. 57; dissected on the same date, negative.

Experiment 14. Pigeon 14 (laboratory-bred) fed with 1796 cysts on 4. vii. 57; five eggs from overnight faeces on 9. vii. 57, twenty-seven on 10. vii. 57, one on 11. vii. 57, five on 12. vii. 57 and one on 13. vii. 57; after that negative till 15. vii. 57; dissected on the same date, negative.

Experiment 15. Pigeon 15, fed with 1250 cysts on 4. vii. 57; faeces negative up to 10. vii. 57; dissected on the same date, negative.

CONCLUSIONS

(1) Domestic pigeons were successfully infected with the tetracotyle stage of *Cotylurus brevis*. Altogether fifteen pigeons were used and ten of them yielded adults of *C. brevis*. Two of the remaining five pigeons were negative, because the tetracotyle introduced were incompletely developed (pigeons, 12, 13). The reasons for the failure to infect the other three are not known.

(2) The egg-laying stage of *Cotylurus brevis* in pigeons is reached in 5 days (pigeons 11, 14) to 6 days (pigeons 2–10) after the introduction of tetracotyle cysts. The adults are usually scattered, sometimes in groups, along the small intestine (pigeons 2, 3, 4, 8, 11); in one case (pigeon 3) they were distributed in two groups of seven and six in the duodenum, while in another pigeon (pigeon 8)

there were groups of five and four in the small intestine; in still another pigeon (pigeon 11) they were in groups of three and two in the small intestine. (Mathias (1925) found the adults of '*Strigea tarda*' attached to the small intestine of ducks, generally in groups of several individuals.)

(3) As is shown by observations on the infestations of pigeons 5, 6, 10, 14, the adults of *Cotylurus brevis* remain in the definitive host for a period of only 2–10 days after the appearance of their eggs in the faeces, after which time the infection disappears. (Mathias (1925), and van Haitsma (1931*a*) referred to a stay in the definitive host of 12–18 days for *Strigea tarda*, and of about 1 week to 11 days for *Cotylurus flabelliformis*.)

(4) The maximum number of eggs collected from the overnight faeces of pigeons 2, 4 and 8 subsequently shown to harbour 2, 2 and 9 adults, was 20, 9 and 3, respectively. It is shown by the examination of the faeces of pigeons 5, 6, 11, 14, that the rate of egg production increases between the second and fourth days of the egg-laying period, after which it decreases again and the parasites are eventually voided in the faeces.

(5) It has been shown by feeding experiments (pigeons 12, 13) that the tetracotyle stage on the twenty-third day of its formation fails to develop into the adult stage; on the other hand infection was successfully introduced (pigeon 6) with 44-day-old tetracotyle.

(6) It was observed commonly that only a very small proportion of the tetracotyle introduced into the pigeons eventually reaches the egg-laying stage. In all, about 20,000 tetracotyle stages were introduced into fifteen pigeons, but only forty-four adult trematodes were recovered, the maximum infection being twenty-six in one pigeon (pigeon 3). At the same time it is pointed out that time did not permit a continuous inspection of all pigeon faeces, and doubtless some adults of *C. brevis* escaped unnoticed in the faeces (a partially decomposed adult was recovered from the overnight faeces of pigeon 7).

Adult

The adult stage of *Cotylurus brevis* has been described in some detail by Dubois & Rausch (1950), it is unnecessary, therefore, to give here a detailed account of the various organ systems. The description given by Dubois & Rausch did not, however, include details of the genitalia. In the present investigation, as a result of a detailed study of sectioned material, the structure of the genitalia proved to be essentially the same as that described by van Haitsma (1931*a*) and La Rue (1932) for *C. flabelliformis* (Faust) and *C. communis* (Hughes), respectively. Measurements of the adults encountered in the present investigations are compared with those of Dubois and Rausch in Table 3.

Adult specimens of *C. brevis* as studied in the present investigation are indistinguishable from those of *C. brevis* as described by Dubois & Rausch; certain very minor points of disagreement, such as the slightly smaller size of specimens described in the present paper and slight differences in the diameter of the germarium are regarded as intraspecific variations.

Of the fifteen known species of *Cotylurus* (fourteen listed by Dubois (1953) and another, *C. strigeoides*, described by Dubois (1958) only four have the testes divided into three posteriorly directed lobes, namely, *C. brevis*, *C. cornutus*, *C. erraticus* and *C. strigeoides*. *C. brevis* may be differentiated from these nearly related species as follows: *C. cornutus* is larger than *C. brevis* and the relative lengths of its two segments are different from those in *C. brevis; C. erraticus* and *C. strigeoides* also, are larger species and the sizes of other internal organs are proportionately larger: in *C. strigeoides* vitelline follicles are present in the posterior part of the fore-body, but this is not so in *C. brevis*.

Table 3. *Measurements (mm.) of* Cotylurus brevis

	C. brevis (present investigation)	*C. brevis* Dubois & Rausch (1950)
Total length	0·728–1·280	1·000–1·800
Fore-body length	0·296–0·504	0·420–0·720
Fore-body width	0·304–0·472	0·330–0·540
Hind-body length	0·544–0·776	0·650–1·080
Hind-body width	0·272–0·480	0·260–0·390
Ratio of the length of hind-body to that of fore-body	c. $1\frac{1}{2}$–2 (1·53–1·83)	1·25–1·94
Oral sucker	0·072–0·121 × 0·061–0·101	0·081–0·120 × 0·072–0·108
Ventral sucker	0·083–0·127 × 0·066–0·134	0·110–0·180 × 0·096–0·170
Pharynx	0·050–0·059 × 0·037–0·044	0·050–0·058 × 0·036–0·145
Germarium	0·072–0·096 × 0·085–0·121	0·075–0·150 × 0·063–0·120
Shape of testes	Anteriorly convex, three large posteriorly directed lobes	0·075–0·150 × 0·053–0·120
Egg capsules	0·088–0·104 × 0·056–0·068	0·091–0·110 × 0·050–0·570

DISCUSSION

The various stages, namely, sporocyst, cercaria, tetracotyle and adult, involved in the life cycle of *C. brevis* have been studied in some detail, but great difficulty was encountered in obtaining a sufficient number of eggs for the rearing of miracidia and the experimental infection of the first intermediate host. The maximum number of eggs ever obtained from the faeces (single overnight sample) of a pigeon was seventy-three.

The genus *Cotylurus* was thoroughly reviewed by Dubois (1938, 1953) who included in it fourteen species, but until the present study of *C. brevis*, the life histories were known for only three of them, namely, *C. cornutus* (Rudolphi, 1808) Szidat (1924, 1929) syn. *Strigea tarda*, (Steenstrup), Mathias (1925) Harper (1931), *Cotylurus communis*, (Hughes), La Rue (1932), syn. *Strigea michiganensis* (La Rue, 1927), syn. *Cotylurus michiganensis* (La Rue), van Haitsma (1930), and *C. flabelliformis* (Faust, 1917), van Haitsma (1931*a*).

Timon-David (1943) also demonstrated a part of the life cycle (from the tetracotyle to the adult) of a species of the genus *Cotylurus* under the name of *C. cornutus* (Rud.), but this was later shown by Dubois & Rausch (1950,) to be *C. brevis*. Since Timon-David's description is very fragmentary, it is not possible to compare adequately the various stages in the life cycle of his '*C. cornutus*' with those of

C. brevis. Timon-David simply fed some tetracotyle stages found in *Lymnaea palustris* to a Chinese teal and recovered adult trematodes which he identified as *Cotylurus cornutus*. Part of the life history of *C. pileatus* (Rudolphi, 1802), Dubois (1937) has also been described by the Erhardt brothers (in Braun, 1894*a*; 1894*b*), who fed *Tetracotyle pileata* (Rud.), Dubois (1938) from the fish *Acerina cernua* to *Larus ridibundus* and got the adults from the bursa fabricii of the host. Recently Copland (1957), in Britain, obtained adults of *Cotylurus erraticus* (Rud. 1809), Szidat (1928) from chicks and pigeons by feeding the tetracotyle stages recovered from the heart of a fish, the Loch Lomond Pown (*coregonus clupeoidis*), but Copland's account is too brief to be commented upon.

Szidat (1929) drew attention to the existence of a holometabolic metamorphosis in the life cycle of *Cotylurus cornutus*, this was confirmed by Harper (1931), and in the present investigation in the life cycle of *C. brevis*. While the present study has provided a detailed investigation into stages in such a holometabolic metamorphosis, no special evidence has been revealed that would contribute to the assessment of the general significance of a holometabolic metamorphosis in the life cycle of a trematode. The situation remains an enigma.

As a special contribution, the present study has demonstrated the persistence through holometabolic metamorphosis of the excretory system, all other systems being disorganized.

Preliminary observations have shown that the tetracotyle stages of *C. brevis*, 44 days after penetration of the cercariae into a secondary intermediate host, are infective, but that those 26 days after penetration are not.

The identification of the adult did not prove too difficult. Since *C. brevis* has testes which are definitely divided into three lobes, a comparison was made only with those species which have trilobed testes. On the basis of this comparison it is concluded that *C. brevis* is very closely related to *C. cornutus*, with which it may be fairly easily confused unless larval stages are taken into consideration.

SUMMARY

1. The life cycle of *Cotylurus brevis* Dubois and Rausch, from the cercaria to the adult, has been investigated for the first time by using laboratory-bred primary, secondary and definitive hosts. The holometabolic metamorphosis with the formation of a tetracotyle stage in a second intermediate host has been described in detail.

2. The cercaria of *C. brevis* obtained from *Lymnaea stagnalis* in Edgbaston Pool has been found to be identical with *Cercaria helvetica XXXIV* Dubois from Lake Neuchâtel. The total number of flame cells in the cercaria is twenty, as opposed to the fourteen in the cercaria of *Cotylurus cornutus* Rudolphi (= '*Strigea tarda*' described by Mathias (1925), Harper (1929, 1931) and Wesenberg-Lund (1934)).

3. In nature the second intermediate host of *Cotylurus brevis* is *Lymnaea stagnalis*. Under experimental conditions *L. pereger* and *L. auricularia* were also found to act as second intermediate hosts, but neither *Planorbis corneus*, *P. carinatus* nor various leeches could act as second intermediate hosts.

4. The tetracotyle stage of *Cotylurus brevis* is morphologically indistinguishable from the corresponding stage of other species of *Cotylurus*.

Part of the work was carried out during the tenure of the Caroline Harrold Research Fellowship of the University of Birmingham and the whole project was supervised by Dr J. Llewellyn.

KEY TO LETTERING OF FIGURES

a.c., anterior canal; *a.l.t.o.*, anterior lip of tribocytic organ; *a.o.*, anterior organ; *a.p.*, apical papilla; *c.*, cilia; *c.l.s.*, cavity of lateral sucking cup; *c.t.o.*, cavity of tribocytic organ; *d.m.*, dorsal muscle; *e.*, eye-spot; *e.p.*, excretory pore; *e.s.p.*, external opening of suctorial pocket; *e.t.o.*, external opening of tribocytic organ; *e.v.*, excretory vesicle; *f.*, flagellet; *f.p.*, forward-pointing spines; *g.*, ganglion; *g.l.s.*, gland of lateral sucking cup; *g.r.*, genital rudiments; *g.t.o.*, gland of tribocytic organ; *i.*, intestine; *i.l.*, inner layer of cyst-wall; *l.s.*, lateral sucking cup; *o.*, operculum; *o.g.l.*, outer gelatinous layer; *o.s.*, oral sucker; *p.*, pharynx; *p.c.*, posterior canal; *p.g.*, penetration gland; *p.l.t.o.*, posterior lip of tribocytic organ; *p.n.*, posterior nerve; *r.t.o.*, rudiments of tribocytic organ; *r.y.*, residual yolk; *s.o.c.*, supra-oesophageal commissure; *s.p.*, suctorial pocket; *s.v.s.*, spines of ventral sucker; *t.e.c.*, transverse excretory commissure; *u.e.s.*, unpigmented eye-spot; *v.m.*, ventral muscle; *v.s.*, ventral sucker.

REFERENCES

Baer, J. G. (1932). A propos du curieux mode de fixation d'un nouveau genre d'hémistomes (trématodes). *Actes Soc. Helvet. Sci. Nat. Thun*, 1932, pp. 386–7.

Brandes, G. P. H. (1890). Die Familie der Holostomiden. *Zool. Jb. Abt. Syst.* **5**, 579–604.

Braun, M. G. C. C. (1894*a*). Helminthologische Notizen. IV. Zur Entwicklungsgeschichte der Holostomiden. *Zbl. Bakt.* **15** (18), 680–2.

Braun, M. G. C. C. (1894*b*). Zur Entwicklungsgeschichte der Holostomiden. Nach den Untersuchungen der Herren Alfred und Oscar Erhardt mitgetheilt. *Zool. Anz.* **17**, 165–7.

Brown, F. J. (1926). Some British freshwater larval trematodes with contributions to their life histories. *Parasitology*, **18** (1), 21–35.

Chandler, A. C. (1954). New Strigeids from Minnesota birds and mammals. *Amer. Midl. Nat.*, **52** (1), 133–41.

Copland, W. O. (1957). The parasites of Loch Lomond fishes. IX, pp. 128–33. In *Studies on Loch Lomond*, by Harry D. Slack.

Cort, W. W. & Brooks, S. T. (1928). Studies on the holostome cercariae from Douglas Lake, Michigan. (Studies on the trematode family Strigeidae (Holostomidae), No. VIII.) *Trans. Amer. Micr. Soc.* **47**, 179–221.

Cuckler, A. C. (1949). Morphological and biological studies on certain strigeid trematodes of mammals. *Summaries of Ph.D. theses, University of Minnesota, Minneapolis, Minn.* **4**, 45–7.

Dubois, G. (1929). Les Cercaires de la région de Neuchâtel. *Bull. Soc. neuchâtel Sci. nat.* **53**, 3–177.

Dubois, G. (1934). Contribution à l'étude des cercaires de la région de Neuchâtel, suivie d'une note sur les cercaires du Lac Noir (Zermatt). *Rev. Suisse Zool.* **41** (2), 73–84.

Dubois, G. (1937). Sur quelques Strigeids. *Rev. Suisse Zool.* **44**, 391–6.

Dubois, G. (1938). Monographie des Strigeida (Trematoda). *Mém. Soc. neuchâtel Sci. nat.* **6**, 1–535.

Dubois, G. (1948). Liste des Strigeides de Suisse, *Rev. Suisse Zool.* **55** (3), 447–76.

Dubois, G. (1953). Systématique des Strigeida. Complément de la monographie. *Mém. Soc. Neuchâtel Sci. Nat.* **8** (2), 1–141.

Dubois, G. (1958). Les Strigeida (Trematoda) de Californie de la collection June Mahon. *Mem. Soc. Neuchâtel Sci. Nat.* **81**, 69–78.

Dubois, G. & Rausch, R. (1950). A contribution to the study of North American Strigeids (Trematoda). *Amer. Midl. Nat.* **43**, 1–31.

Faust, E. C. (1917). Notes on the cercariae of Bitter Root Valley, Montana. *J. Parasit.* **3**, 105–23.

FAUST, E. C. (1918*a*). Life history studies on Montana Trematodes. *Ill. Biol. Monogr.* **4** (1), 7–120.

FAUST, E. C. (1918*b*). The anatomy of *Tetracotyle iturbei* Faust, with a synopsis of described tetracotyliform larvae. *J. Parasit.* **5**, 69–79.

FAUST, E. C. (1922). Phases in the life history of a holostome, *Cyathocotyle orientalis*, nov.spec., with notes on the excretory system of the larva. *J. Parasit.* **8**, 78–85.

HARPER, W. F. (1929). On the structure and life histories of British freshwater larval trematodes. *Parasitology*, **21**, 189–219.

HARPER, W. F. (1931). On the structure and life history of British freshwater furcocercariae. *Parasitology*, **23**, 310–24.

HUGHES, R. C. (1927). Studies on the Trematode family Strigeidae (Holostomidae) No. VI. A new metacercaria *Neascus ambloplitis* sp.n.v., representing a new larval group. *Trans. Amer. Micr. Soc.* **47**, 248–67.

HUGHES, R. C. (1928*a*). Studies on the Trematode family Strigeidae (Holostomidae) No. VII. *Tetracotyle pipientis* Faust. *Trans. Amer. Micr. Soc.*, **47**, 42–53.

HUGHES, R. C. (1928*b*). Studies on the Trematode family Strigeidae (Holostomidae) No. IX. *Neascus van-cleavei* (Agersborg). *Trans. Amer. Micr. Soc.*, **47**, 320–41.

HUGHES, R. C. (1928*c*). Studies on the Trematode family Strigeidae (Holostomidae) No. XIII. Three new species of Tetracotyle. *Trans. Amer. Micr. Soc.* **47**, 414–33.

HUGHES, R. C. (1929). Studies on the Trematode family Strigeide (Holostomidae) No. XVII. *Tetracotyle flabelliformis* Faust. *Pap. Mich. Acad. Sci.* **10**, 495–508.

ILES, C. (1952). The larval trematodes of certain freshwater molluscs. I. The Furcocercaria. *Parasitology*, **49**, 478–504.

KRAUSE, R. K. L. (1914). Beiträge zur Kenntnis der Hemistominen. *Z. wiss. Zool.* **112**, 93–238.

LA RUE, G. R. (1927). A new species of Strigea from the herring gull, *Larus argentatus* (Pont) with remarks on the function of the holdfast organ. *J. Parasit.* **13**, 226.

LA RUE, G. R. (1932). Morphology of *Cotylurus communis* Hughes (Tremadoda: Strigeidae). *Trans. Amer. Micr. Soc.* **51**, 28–47.

MATHIAS, P. (1925). Recherches expérimentales sur le cycle évolutif de quelques trématodes. *Bull. Biol.* **59**, 1–123.

MUEHLING, P. (1898). Die Helminthenfauna der Wirbeltiere Ostpreussens. *Arch. Naturg., Berl.*, **64**, 1–118.

NAJARIAN, H. H. (1954). Developmental stages in the life cycle of *Echinoparyphium flexum* (Linton, 1892) Dietz, 1910 (Trematoda: Echinostomatidae). *J. Morph.* **94**, 165–89.

OLIVIER, L. J. & CORT, W. W. (1941). *Cercaria douglasi* Cort, 1917, and its relation to the cercaria of *Cotylurus flabelliformis* Faust, 1917. *J. Parasit*, **27**, 343–6.

OLIVIER, L. J. & CORT, W. W. (1942). An experimental test of the life cycle described for *Cotylurus communis* (Hughes). *J. Parasit.* **28**, 75–81.

PEARSON, J. C. (1956). Studies on the life cycles and morphology of the larval stages of *Alaria arisaemoides* Augustine and Uribe, 1927, and *Alaria canis* La Rue and Fallis, 1936 (Trematoda: Diplostomidae). *Canad. J. Zool.*, **34** (4), 295–387.

PEARSON, J. C. (1959). Observations on the morphology and life cycle of *Strigea elegans* Chandler and Rausch, 1947 (Trematoda: Strigeidae). *J. Parasit.* **45** (No. 2), 155–74.

PETERSON, R. T., *et al.* (1954). *Field Guide to the Birds of Britain and Europe.* Collins.

REES, F. G. (1932). An investigation into the occurrence, structure and life histories of the trematode parasites of four species of *Limnaea* L. *truncatula* (Müll.), *L. pereger* (Müll.), *L. palustris* (Müll.) and *L. stagnalis* (Linné) and *Hydrobia jenkinsi* (Smith) in Glamorgan and Monmouth. *Proc. Zool. Soc. Lond.* **1**, 1–32.

REES, F. G. (1939). Studies on the germ cycle of the digenetic trematode *Parorchis acanthus* Nicoll. Part 1. Anatomy of the genitalia and gametogenesis in the adult. *Parasitology*, **31** (4), 417–33.

REES, F. G. (1940). Studies on the germ cycle of the digenetic trematode *Parorchis acanthus* Nicoll. Part II. Structure of the miracidium and germinal development in the larval stages. *Parasitology*, **32** (4), 372–91.

REES, F. G. (1952). The structure of the adult and the larval stages of *Plagiorchis* (*Multiglandularis*) *megalorchis* N. Nom. from the Turkey and experimental demonstration of the life history. *Parasitology*, **42**, 92–113.

Rees, F. G. (1955). The adult and diplostomulum stage (*Diplostomulum phoxini* Faust) of *Diplostomum pelmatoides* Dubois and an experimental demonstration of part of the life cycle. *Parasitology*, **45**, 295–312.

Rees, F. G. (1957). Cercaria *diplostomi phoxini* Faust, a furcocercaria which develops into *Diplostomulum phoxini* in the brain of the minnow. *Parasitology*, **47**, 126–37.

Szidat, L. (1924). Beiträge zur Entwicklungsgeschichte der Holostomiden. *Zool. Anz.* **58**, 299–314.

Szidat, L. (1928). Zur Revision der Trematodengattung *Strigea* Abildgard. *Z. Bakt.* I. *Abt.* **105**, 204–15.

Szidat, L. (1929*a*). Beiträge zur Kenntnis der Gattung *Strigea* (Abild). 1. Allgemeiner Teil: Untersuchungen über die Morphologie, Physiologie and Entwicklungsgeschichte der Holostomiden nebst Bemerkungen über die Metamorphose der Trematoden und die Phylogenie derselben. *Z. Parasitenk.* **1**, (4–5), 612–87.

Szidat, L. (1929*b*). Beiträge zur Kenntniss der Gattung *Strigea* (Abild.). II. Spezieller Teil: Revision der Gattung *Strigea* nebst Beschreibung einer Anzahl neuer Gattung und Arten. *Z. Parasitenk.* **1**, (4–5), 688–764.

Szidat, L. (1931). Beiträge zur Entwicklungsgeschichte der Holostomiden. IV. Die cercariae des Entenparasiten *Apatemon* (*Strigea*) *gracilis* Rud. und ihre Entwicklung im Blutgefässsystem des Zwischenwirtes (*Herpobdella atomaria* Car.). *Z. Parasitenk.* **3** (2), 160–72.

Timon-David, J. (1943). Contributions à l'étude biologique de La Camargue. Parasitologie I. Sur la présence en Camargue et le développement expérimental de *Cotylurus cornutus* (Rud.) (trémadode, strigéidé). *Bull. Mus. Hist. nat. Marseille*, **3** (1), 17–21.

Tubangui, M. A. (1933). Trematode parasites of Philippine Vertebrates. VI. Descriptions of new species and classification. *Philipp. J. Sci.*, **52** (2), 157–97.

Van Haitsma, J. P. (1930). Studies on the Trematode family Strigeidae (Holostomidae). No. XXI. Life cycle and description of the cercaria of *Cotylurus michiganensis* (La Rue). *J. Parasit.* **16**, 224–30.

Van Haitsma, J. P. (1931*a*). Studies on the Trematode family Strigeidae (Holostomidae). XXII. *Cotylurus flabelliformis* (Faust) and its life history. *Pap. Mich. Acad. Sci.* **13**, 447–482.

Van Haitsma, J. P. (1931*b*). Studies on the Trematode family Strigeidae (Holostomidae). XXIII. *Diplostomum flexicaudum* (Cort & Brooks) and stages in its life history. *Pap. Mich. Acad. Sci.* **13**, 447–516.

Wesenberg-Lund, C. (1934). Contributions to the development of the Trematoda Digenea. Part II. The biology of the freshwater Cercariae in Danish freshwaters. *K. danske Vidensk. Selsk. Naturv. og Math. Afd.*, **9**, **5** (3), 1–223.

Parasitology (1960), **50**, 577–580
With 1 *text-figure*
Printed in Great Britain

Shunsennia gracilis, a new species of Trombiculid Mite from Korea (Acarina, Trombiculidae)*

By HYONG-SUN AH†
Department of Preventive Medicine, Sudo Medical College, Seoul, Korea

(*Received* 20 *February* 1960)

The genus *Shunsennia*, with *S. tarsalis* Jameson & Toshioka as the type, was first established by Jameson & Toshioka (1953) for the chiggers collected from a red-backed vole *Clethrionomys rufocanus regulus* (Thomas) and a field mouse *Apodemus agrarius coreae* Thomas from South Korea. To this genus they also transferred *Hannemania ochotona* Radford, 1942.

Teller (1956) described *Shunsennia biplumulosa* from a Japanese shrew-mole, *Urotrichus talpoides* and provided a key for the identification of three species of the genus. He amended the generic criteria of *Shunsennia* to include 'the presence or absence of ventral cheliceral teeth, and a palpal claw with two to four prongs'.

The new species described herein clearly fits Teller's emendation of the generic description of *Shunsennia*. It is somewhat similar to *Parashunsennia harunaensis*, Kumada, 1956, a monotypic genus. However, the presence of eyes and ventral cheliceral teeth, and the absence of pseudochela in the new species places it in the genus *Shunsennia*.

The new species recorded in this paper is apparently the second species of the genus to be found in Korea and comprises a fourth member of this genus. So far, the known geographical distribution of *Shunsennia* is characteristically restricted to the areas of Chunchon and Wonju in Korea.

Shunsennia gracilis sp.nov.

Body. Colour in life whitish. Eyes relatively small, inconspicuous, 2/2 without ocular plate. Striae conspicuous. Length and width of holotype, partially engorged, 465 by 310μ. Anus located in posterior third of venter.

Gnathosoma. Cheliceral base with numerous, small punctae. Chelicera with numerous minute teeth (more than 12) on ventral surface of cheliceral blade and a subapical dorsal tooth. Palpal coxal, femoral, genual, and dorsal tibial setae feathered; lateral and ventral tibial setae nude. Palpal claw two-pronged; axial prong ventral. Palpal tarsus with seven feathered setae and a spur (its length about equal to the width of the tarsus). Galeal setae nude.

Scutum. Broadly trapezoidal, about twice as wide as long, anterior border with marked mesal convexity, posterior border slightly mesally concave. Punctae

* This investigation was supported in part by a research grant N.38 from the Division of Medical Research and Development, Office of Surgeon General, Republic of Korea Army.
† Capt., V.C., R.O.K. Army.

numerous. Anteromedian setae paired, their length about equal to the antero-lateral setae. Sensillary bases in advance of a line connecting postero-lateral setae. Sensillae nude. Scutal measurements of holotype and paratype (in microns) are as follows:

	AW	PW	SB	ASB	PSB	AP	AM	AL	PL	S
Holotype:	93	118·5	42	42·7	15·8	40	94	93	87	87
Paratype:	91	118·5	42	42·7	15	39·8	82·5	78·8	86·3	90

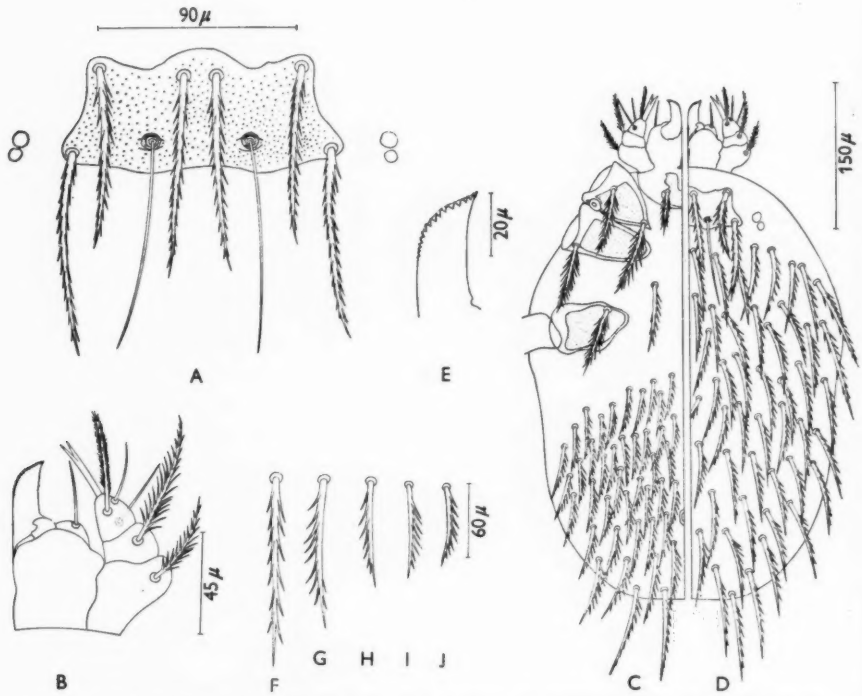


Fig. 1. *Shunsennia gracilis*, sp.nov., A, scutum; B, dorsal aspect of right half of gnathosoma, palpal tarsus omitted; C, venter; D, dorsum; E, chelicera; F, dorsal lateral seta; G, dorsal posterior seta; H. dorsal anterior seta; I and J, ventral setae.

Legs. Leg I, 370μ; II, 390μ; III, 430μ. Setae distributed as follows: Leg I; coxa, 2 plumose setae; trochanter, 1 plumose seta; femur, 6 plumose setae; genu, 4 plumose setae, 1 genuala, 1 microgenuala; tibia, 8 plumose setae, 2 tibialae, 1 microtibala; tarsus, 31 plumose setae, 1 spur, 1 microspur, 1 subterminala, 1 pretarsala. Leg II: coxa, 1 plumose seta; trochanter, 1 plumose seta; femur, 5 plumose setae; genu, 4 plumose setae, 1 genuala, 1 microgenuala; tibia, 7 plumose setae, 2 tibialae; tarsus, 22 plumose setae, 1 spur, 1 microspur, 1 pretarsala. Leg III: coxa, 1 plumose seta; trochanter, 1 plumose seta; femur, 4 plumose setae, 1 genuala; tibia, 7 plumose setae, 1 tibiala; tarsus, 20 plumose setae. Each tarsus with two claws and a claw-like empodium.

Body setae. Dorsal setae in irregular rows, similar in character to the posterolateral setae of scutum, about 80μ, caudads and laterals longer; dorsal setal formula for holotype is 17–15–14–14–12–8–6–4–2. Ventral setae 0–2 (sternals) plus about 84, those anterior to the anus smaller than the dorsal setae; while those posterior to the anus resemble the dorsal setae. The arrangement of ventral setae obscure. A single pair of sternal setae located between coxa III. All measurements are given in microns.

Type data. Holotype from *Apodemus speciosus peninsulae* Thomas, Mt. Samak (654 m. elevation), 3 miles west of Chunchon, Korea on the 27 March 1958, H.-S. Ah, Collector. Paratype from *A. agrarius coreae* Thomas from the type locality, 4 May 1958, H.-S. Ah, Collector. Types are deposited in the author's collections.

Remarks. Shunsennia gracilis resembles *Chatia setosa* Brennan, 1946, in having cheliceral teeth, but exhibits empodia on all tarsi, the absence of which is characteristic of *Chatia* (Brennan, 1946, 1955). *Shunsennia gracilis* also closely resembles the generic description of *Parashunsennia* as given by Kumada (1956) in all but the presence of eyes and ventral cheliceral teeth, and the absence of pseudochela.

Shunsennia gracilis is readily distinguishable from the other three members of this genus in having many minute ventral teeth on cheliceral blade along with other distinctive characteristics of the body. *S. gracilis* is most closely allied to *S. biplumulosa* Teller, 1956. However, it can be easily separated from the latter species chiefly by the number of minute ventral teeth on the cheliceral blade, the number of coxal setae and the character of ventral tibial setae. *S. gracilis* has more than twelve minute ventral teeth on the cheliceral blade which is considerably more than in *S. biplumulosa.*

Key to the species of Shunsennia *Jameson & Toshioka*

1. Minute teeth on ventral surface of cheliceral blade; one pair of sternal setae; palpal claw two-pronged. **2**

   No minute teeth on ventral surface of cheliceral blade; two or three pairs of sternal setae; palpal claw three-to four-pronged*. **3**

2. Five minute ventral teeth on cheliceral blade; ventral tibial setae with short barbs; dorsal setae, about 60 to 63 in number; coxal setae 2-2-2. **biplumulosa** Teller, 1956

   More than twelve minute ventral teeth on cheliceral blade; ventral tibial setae nude; dorsal setae about 90 or more; coxal setae 2-1-1. **gracilis** sp.nov.

3. Three pairs of sternal setae; usually three setae on coxa III; sensillae barbed basally; palpal claw with four prongs. **ochotona** Radford, 1942.

   Two pairs of sternal setae; one seta on coxa III; sensillae without basal barbs. **tarsalis** Jameson & Toshioka, 1953

SUMMARY

Shunsennia gracilis is described as a new species from a wood mouse, *Apodemus speciosus peninsulae* Thomas and a field mouse, *A. agrarius coreae* Thomas, of Mt Samak in the vicinity of Chunchon, Korea. A key to the *Shunsennia* is presented.

* A microscopic study of a series of specimens (125 individuals) of *Shunsennia tarsalis* has shown that the Korean specimens definitely possess the three-pronged palpal claw as originally described by Jameson & Toshioka (1953), whereas only two-pronged palpal claw is indicated in Sasa's (1956) description of the Japanese specimens of *S. tarsalis.*

I am grateful to Professor I. H. Choo of the Department of Preventive Medicine and Hygiene, Sudo Medical College, Seoul, for his great interest and encouragement during the course of this study, to Dr B. S. Seo of the Department of Parasitology, Seoul University Medical College, and to Dr H. Y. Chung of the National Institute of Health, Seoul, for their aid in obtaining literature. I also wish to thank Dr Gordon Field, Major, MSC, U.S.A., for his continued encouragement and guidance during the period of my assignment to 37th Medical Company (PM) (Sep), APO 301, San Francisco, California.

REFERENCES

BRENNAN, J. M. (1946). A new genus and species of Chigger. *Chatia setosa* (Trombiculidae, Acarina) from northwestern U.S. *J. Parasit.* **32**, 132–5.

BRENNAN, J. M. (1955). *Chatia* Brennan, 1946: A new generic character and additional notes (Acarina, Trombiculidae). *J. Parasit.* **41**, 283–4.

JAMESON, E. W. & TOSHIOKA, S. (1953). *Shunsennia tarsalis*, a new genus and species of Chigger from Korea (Acarina, Trombiculidae). *Proc. Biol. Soc. Wash.* **66**, 89–92.

KUMADA, N. (1956). *Parashunsennia harunaensis*, a new genus and species of Trombiculid Mite from Japan. *Bull. Tokyo Med. Dent. Univ.* **2** (3), 257–61.

RADFORD, C. D. (1942). The larval Trombiculinae (Acarina: Trombiculidae) with description of twelve new species. *Parasitology*, **34**, 55–81.

SASA, M. (1956). Tsutsugamushi and Tsutsugamushi Disease, pp. 159–62.

TELLER, L. W. (1956). *Shunsennia biplumulosa*, a new Species of Chigger from Japan (Acarina: Trombiculidae). *J. Parasit.* **42** (2), 173–5.

WHARTON, G. W., JENKINS, D. W., BRENNAN, J. M., FULLER, H. S., KOHLS, G. M. & PHILIP, C. B. (1951). The terminology and classification of trombiculid mite (Acarina, Trombiculidae). *J. Parasit.* **37** (1), 13–31.

Parasitology (1960), **50**, 581–585
With 9 text-figures
Printed in Great Britain

On a new haplosporidian (protozoa) *Nephridiophaga xenoboli* in the gut of a millipede *Xenobolus carnifex*

By P. N. GANAPATI and C. C. NARASIMHAMURTI*
Department of Zoology, Andhra University, Waltair, India

(*Received* 30 *September* 1959)

INTRODUCTION

While examining the sections of the gut of a millipede, *Xenobolus carnifex* in search of developmental stages of a cephaline gregarine we frequently came across large numbers of plasmodia in the epithelium which proved to be stages in the life history of a haplosporidian. While haplosporidian parasites have previously been reported from a variety of hosts such as annelids, nemertines, molluscs, amphipods, rotifers, trematodes and insects, this appears to be the first record of the parasite from a myriapod host.

Caullery & Mesnil (1899) created the class Haplosporidia to include a homogeneous assemblage of protozoan parasites from annelids whose characteristic feature was the simplicity of the spore structure. The same authors added a few other less well-known genera to this group and more forms were added by subsequent workers. The group as at present constituted (Grassé, 1953) includes five well-defined genera, *Haplosporidium*, *Urosporidium*, *Aneurosporidium*, *Phycosporidium* and *Nephridiophaga*. Seven other genera whose systematic position is at present uncertain are treated as an appendix to this group.

For reasons discussed elsewhere in the text of the paper the present form is considered a new species belonging to the genus *Nephridiophaga* for which the name *N. xenoboli* sp.nov. is proposed.

MATERIAL AND METHODS

Millipedes collected from underneath the debris of cashew-nut plants and felled plantain trees were kept isolated in glass finger-bowls with moist blotting paper at the bottom to keep the faecal matter passed out in a humid condition. The gut contents and the faeces were examined under a microscope for the developmental stages of the parasite. Smears of the gut contents and the faeces containing the cysts were fixed in hot Schaudinn's fluid and stained with Heidenhain's iron haematoxylin. Feulgen's stain was also used after fixation in Carnoy's solution. The gut of the millipede was fixed in alcoholic Bouin's fluid for 1 hr. at 60° C. and overnight at room temperature. Sections were cut 5μ thick and stained with iron haematoxylin and Feulgen stains.

Infection experiments were carried out by feeding clean hosts with spores of the

* Part of the Thesis approved for the D.Sc. Degree of the Andhra University.

parasite mixed with corn-flour. The clean hosts were judged as such when repeated examination of the faecal matter was negative for the presence of the spores of the haplosporidian.

OBSERVATIONS ON THE PARASITE

Amoebula

The life cycle of the parasite starts in the form of a minute amoebula occurring free in the lumen of the gut (Fig. 2). It measures $4{\cdot}5 \times 4{\cdot}0\mu$ and has a centrally placed nucleus. The cytoplasm is uniformly hyaline. In fresh smears of the gut contents the amoebula is seen to put forth a single blunt pseudopodium and to move in jerks. In sections such small amoeboid stages have been observed to be in close apposition to the epithelium and presumably they may gain entry into it by some enzymic action. All the subsequent developmental stages in the life-history of the parasite, till the spores are formed, are intra-epithelial. Cysts containing the spores are liberated in large numbers into the lumen of the gut by rupture of the epithelium.

Intracellular stages

The earliest intracellular stages observed had three nuclei and the parasite measured $6 \times 4\mu$ (Fig. 1). The cytoplasm is granular and stains deep with iron haematoxylin. The nuclei are in the form of rings of chromatin surrounded by a clear space which appears as a halo. These parasites grow into the multinucleate plasmodia (Figs. 3–5). It has not been possible to follow the details of nuclear division. Some dividing stages of the nuclei suggest a kind of amitosis, with two clumps of chromatin at either pole connected by a thin strand of the same material. A typical spindle or chromosomes have not been observed either in haematoxylin or in Feulgen preparations. The nuclear division continues until the plasmodium has as many as 100–150 nuclei. When fully developed the plasmodium may be rounded, cylindrical or elliptical depending upon the direction of the growth of the parasite. The largest observed measured $32 \times 18\mu$ and was oval in shape. Large numbers of multinucleate plasmodia are found in groups inside the epithelium of the gut. The nuclei retain their ring-shaped structure, as in the earlier stages and each nucleus is surrounded by a clear halo. The cytoplasm stains deep with iron haematoxylin and is coarsely granular.

The increase in the number of plasmodia may presumably take place through plasmotomy resulting in smaller plasmodia which may grow again or give rise to uninucleate bodies. Ultimately both the larger plasmodia and the smaller ones produce through a process of endogenous budding large numbers of uninucleate bodies which are the gametes (Fig. 7) measuring $2{\cdot}5 \times 2{\cdot}0\mu$. The nucleus, which is situated eccentrically at one pole, is compact, homogeneously staining and surrounded by a clear space. The cytoplasm is hyaline without any inclusions. Along with these uninucleate bodies there are also binucleate bodies which appear to be formed by fusion of two of the uninucleate bodies. If they had been stages preceding the formation of the uninucleate bodies, the nucleus should show the same type of ring structure as seen in the nuclei of the plasmodium instead of the compact nucleus of the gametes. In the same group can also be seen other types

of uninucleate bodies which are larger in size, and have a greater quantity of chromatin material. These are the zygotes formed as a result of conjugation and fusion of two gametes. The zygote develops into sporoblasts and ultimately into

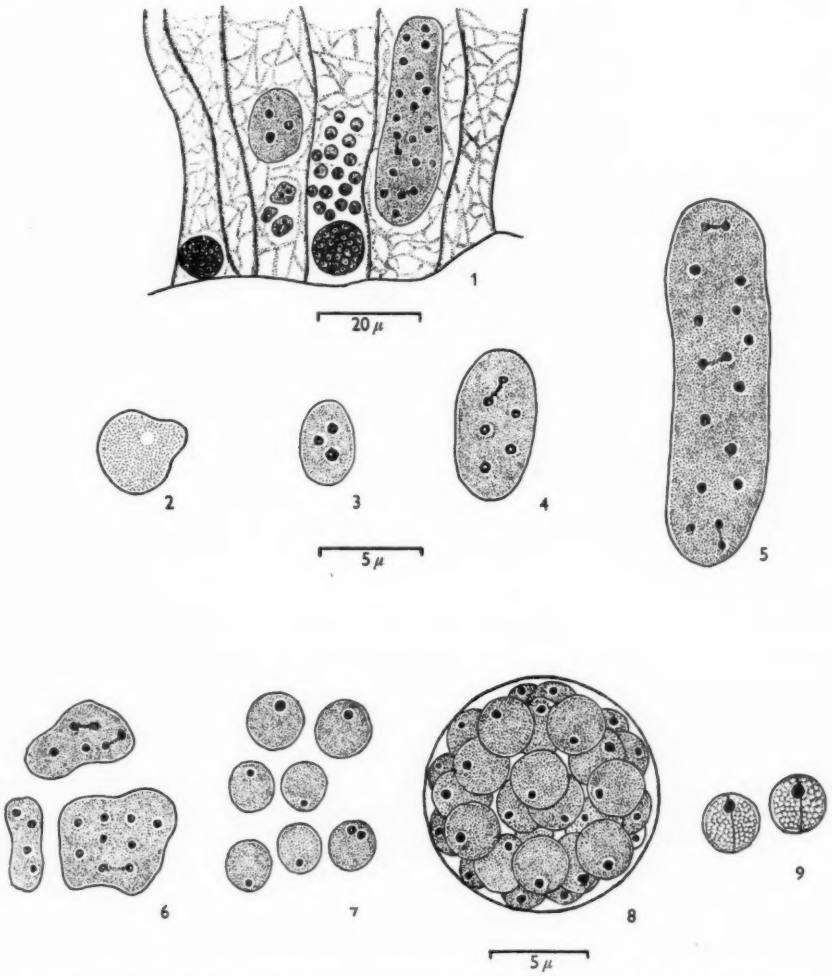


Fig. 1. Part of the gut epithelium showing the different stages in the life-history of the haplosporidian, *Nephridiophaga xenoboli* sp.n.

Fig. 2. Amoebula.

Fig. 3. Plasmodium with few nuclei.

Figs. 4, 5. Fully grown plasmodium.

Fig. 6. Smaller plasmodia produced as a result of plasmotomy.

Fig. 7. Uninucleate gametes and the binucleate sporoblasts.

Fig. 8. A cyst enclosing the spores.

Fig. 9. Mature spore showing the bivalve nature of the spore wall.

spores. When the sporogony is at an advanced stage the zygotes and the sporoblasts aggregate towards the centre of the plasmodium, the residual cytoplasm shrinks considerably, and most of it disintegrates. A tough, thin, transparent membrane is formed enclosing these bodies which are the 'cysts'.

The cysts present a mulberry-like appearance (Fig. 8), and lie within the epithelium of the host's gut. They are released into the lumen by the rupture of the epithelium, whence thay pass out with the faeces.

The typical spore is perfectly spherical and transparent measuring $3{\cdot}0 \times 3{\cdot}0\mu$ (Fig. 9). The two valves are fused along the longitudinal axis and a suture can be made out at the line of fusion. There is no trace of a second envelope in the spore nor are there any prominences on the spore wall as in the case of *Haplosporidum*. It is difficult to make out the internal structure of the spores in fresh preparations. In stained preparations each spore appears perfectly spherical and the nucleus is situated eccentrically. It is homogeneously stained with iron haematoxylin and Feulgen stains, and is surrounded by a clear halo. A clear nuclear membrane could not be seen. Though a large number of spores and sporoblasts in the fresh and stained conditions have been examined, we have not been able to observe any process comparable to autogamy as reported for *Coelosporidium periplanetae* (Sprague, 1940).

The sporogony in the present form appears to be similar to that observed by Granata (1914) in *Haplosporidium limnodrili*, where the plasmodium resolves itself into uninucleate gametes, which fuse in pairs resulting in sporoblasts which later transform themselves into spores. Similar observations were also made for *H. chitonis* by Pixell-Goodrich (1915).

Experimental infection has been produced successfully by feeding the spores to healthy hosts, which started passing spores in about 30–35 days. Natural infection probably takes place when the spores of the haplosporidian from an infected host are ingested with the food material by a fresh host. Infection is generally widespread and nearly 80% of the hosts examined showed infection. Not only the percentage but the intensity of infection may sometimes be so heavy that the possibility of auto-infection cannot be excluded.

SYSTEMATIC POSITION

The genus and type species, *Nephridiophaga apis*, were created by Ivanić (1937) for a haplosporidian parasite which he found in the epithelial cells of the malpighian tubules of the honey bee, *Apis mellifica*. An important character of the genus, according to the above author, is its intracellular development. The close similarity in the life history of the above parasite and that of *Coelosporidium periplanetae*, from the cockroach, *Periplaneta americana*, supported by the observations of Debaisieux (1927) of the occurrence of an intracellular development in *Coelosporidium periplanetae*, led Ivanić (1937) to transfer the former to the genus *Nephridiophaga* and name it *N. periplanetae*.

Sprague (1940), however, was not in favour of this transfer and in his opinion 'the presence of intracellular stages in neither of the species has been conclusively

demonstrated, for the smear method alone on which Ivanić based his observations does not seem adequate'. Sprague studied sectioned material of *Coelosporidium periplanetae* but was not able to observe any intraepithelial stages. He did not however, examine sectioned material of *Nephridiophaga apis* and until this is done the intracellular development of *N. apis* cannot be doubted.

The present parasite in the gut of the millipede *Xenobolus carnifax* is clearly a haplosporidian which, on account of its intracellular development and the simple bivalve nature of the spore, could be assigned to the genus *Nephridiophaga*. The occurrence in a millipede host appears to be sufficient reason to describe it as a new species of the genus, viz. *N. xenoboli* sp.nov.

SUMMARY

1. The morphology and life history of a new haplosporidian parasite, *Nephridiophaga xenoboli* sp.nov. from the gut of a millipede, *Xenobolus carnifex* are described.

2. The earliest stage hatched from the spore is a free uninucleate amoebula which penetrates the gut epithelium and grows into a multinucleate plasmodium.

3. The uninucleate gametes are formed by a process of endogeneous budding in the centre of the plasmodium and the sporoblast is formed as a result of fusion of two such gametes.

4. A characteristic cyst membrane enclosing about forty to fifty sporoblasts is formed immediately after fertilization is completed which is accompanied by a disintegration of the peripheral layer of the cytoplasm of the plasmodium.

5. Each sporoblast develops into a bivalved simple spherical spore, 3·0μ in diameter.

REFERENCES

Caullery, M. & Mesnil, F. (1899). Sur le genre *Aplosporidium* (nov.) et l'ordre nouveau des Aplosporidies. *C.R. Soc. Biol., Paris*, **51**, 789.

Debaisieux, P. (1927). A propos des Cnidosporidies des blattides. *C.R. Soc. Biol., Paris*, **96**, 1404.

Granata, L. (1913). Ciclo di sviluppo di *Haplosporidium limnodrili* n.sp. *R.C. Accad. Lincei*. **22**, 734.

Granata, L. (1914). Ricerche sul ciclo evolutivo di *Haplosporidium limnodrili* Granata. *Arch. Protistenk*. **35**, 47.

Grassé, P. P. (1953). *Traité de Zoologie*, Tome I, Fasc. I. Paris: Masson and Cie.

Ivanić, M. (1937). Die Entwicklungsgeschichte und die parasitäre Zerstörungsarbeit einer in den Malpigischen Gefässe der Honigbiene (*Apis mellifica* L.) schmarotzenden Haplosporidie, *Nephridiophaga apis* gen.n., spec. nov. *La Cellule*, **45**, 291.

King, S. D. (1926). Cytological observations on *Haplosporidium* (*Minchinia*) *chitonis*. *Quart. J. Micr. Sci*. **70**, 147.

Pixell-Goodrich, H. (1915). *Minchinia*, a Haplosporidian. *Proc. Zool. Soc. Lond*. **85**, p. 445.

Sprague, V. (1940). Observations on *Coelosporidium periplanetae* with special reference to the development of the spore. *Trans. Amer. Micr. Soc*. **59**, 460.

Index of Authors

AH, HYONG-SUN. *Shunsennia gracilis*, a new species of Trombiculid mite from Korea (Acarina, Trombiculidae). (With 1 Figure in the Text), 577

AHLUWALIA, S. S. *See* PANDE, B. P.

ARTHUR, D. R. A review of some ticks (Acarina: Ixodidae) of sea birds. Part II. The taxonomic problems associated with the *Ixodes auritulus-percavatus* group of species. (With 78 Figures in the Text), 199

ARTHUR, D. R. A review of some ticks (Acarina: Ixodidae) of sea birds. Part III. A re-description of the male of *Ixodes kerguelenensis* André & Colas-Belcour, 1942. (With 8 Figures in the Text), 227

BAKER, J. R. Trypanosomes and dactylosomes from the blood of fresh-water fish in East Africa. (With 1 Plate and 2 Figures in the Text), 515

BAUGH, SANJIB CHANDRA. Studies on Indian Rhynchobdellid leeches. I. (With 6 Figures in the Text), 287

BISHOP, ANN and MCCONNACHIE, ELSPETH W. Further observations on the *in vitro* development of the gametocytes of *Plasmodium gallinaceum*, 431

BRAMBELL, M. R. *See* FIELD, A. C.

CAMPBELL, J. ALLAN. *See* FIELD, A. C.

CORLISS, J. O. *Tetrahymena chironomi* sp.nov., a ciliate from midge larvae, and the current status of facultative parasitism in the genus *Tetrahymena*. (With 7 Figures in the Text), 111

COSTA, M. *See* THEODOR, O.

DASGUPTA, B. Lipids in different stages of the life cycles of malaria parasites and some other sporozoa. (With 2 Figures in the Text), 501

DASGUPTA, B. Polysaccharides in the different stages of the life-cycles of certain sporozoa. (With 3 Figures in the Text), 509

DINNIK, J. A. and DINNIK, N. N. Development of *Carmyerius exoporus* Maplestone (Trematoda: Gastrothylacidae) in a snail host. (With 8 Figures in the Text), 469

DINNIK, N. N. *See* DINNIK, J. A.

EUZET, L. and WILLIAMS, H. HARFORD. A re-description of the trematode *Calicotyle stossichii* Braun, 1899, with an account of *Calicotyle palombi* sp.nov. (With 11 Figures in the Text), 21

FIELD, A. C., BRAMBELL, M. R. and CAMPBELL, J. ALLAN. Spring rise in faecal worm-egg counts of housed sheep, and its importance in nutritional experiments. (With 3 Figures in the Text), 387

GANAPATI, P. N. and NARASIMHAMURTI, C. C. On a new haplosporidian (protozoa) *Nephridiophaga xenoboli* in the gut of a millipede *Xenobolus carnifex*. (With 9 Figures in the Text), 581

GRESSON, R. A. R. *See* PANTELOURIS, E. M.

HANUMANTHA-RAO, K. Studies on *Penetrocephalus ganapatii*, a new genus (Cestoda: Pseudophyllidea) from the marine teleost *Saurida tumbil* (Bloch). (With 1 Plate and 8 Figures in the Text), 155

HANUMANTHA-RAO, K. The problem of Mehlis's gland in helminths with special reference to *Penetrocephalus ganapatii* (Cestoda: Pseudophyllidea), 349

HARRINGTON, J. S. A simple apparatus for the artificial feeding of *Rhodnius prolixus* (Hemiptera, Reduviidae), 273

HARRINGTON, J. S. Studies on *Rhodnius prolixus*: growth and development of normal and sterile bugs, and the symbiotic relationship, 279

ILES, CHRISTINE. The larval trematodes of certain fresh-water molluscs. (With 1 Plate), 401
INGRAM, D. G. and SOLTYS, M. A. Immunity in trypanosomiasis. Part IV. Immuno-conglutinin in animals infected with *Trypanosoma brucei*, 231

JACK, K. M. *Geckobia japonicum*, a re-publication of *Pterygosoma japonicum* Kishida, 1927. (Acarina, Pterygosomidae). (With 1 Figure in the Text), 89
JAIN, G. P. On the genus *Artyfechinostomum* (Lane, 1915) (Echinostomatidae: Trematoda), 1
JAIN, G. P. Further observations on *Artyfechinostomum mehrai* (Faruqui) (Echinostomatidae: Trematoda). (With 6 Figures in the Text), 7
JOHRI, G. N. A new paruterinid cestode, *Lallum magniparuteria* gen. et sp.nov. from the intestine of a common teal, *Nettion crecca* Linn, 269

KULASIRI, C. The indicator system of the Sabin–Feldman phenomenon in *Toxoplasma*, 13
KULASIRI, C. The nature of the cytoplasm-modifying antibodies. (With 2 Figures in the Text), 419

LEE, D. L. The effect of changes in the osmotic pressure upon *Hammerschmidtiella diesingi* (Hammerschmidt, 1838) with reference to the survival of the nematode during moulting of the cockroach. (With 4 Figures in the Text), 241
LEE, D. L. The distribution of glycogen and fat in *Thelastoma bulhõesi* (Magalhães, 1900), a nematode parasitic in cockroaches. (With 24 Figures in the Text), 247
LLEWELLYN, J. and OWEN, I. L. The attachment of the monogenean *Discocotyle sagittata* Leuckart to the gills of *Salmo trutta* L. (With 1 Plate and 4 Figures in the Text), 51

MAWSON, PATRICIA M. Two new species of trichostrongyle nematodes from the Australian pouched mouse, *Phascogale flavipes* (Waterhouse). (With 11 Figures in the Text), 425
MCCONNACHIE, ELSPETH W. Experiments on the encystation of *Opalina* in *Rana temporaria*, 171
MCCONNACHIE, ELSPETH W. *See* BISHOP, ANN
MINES, J. J. *See* SPRENT, J. F. A.

NARASIMHAMURTI, C. C. *See* GANAPATI, P. N.
NASIR, P. Trematode parasites of snails from Edgbaston Pool: the life history of the strigeid *Cotylurus brevis* Dubois & Rausch, 1950. (With 8 Figures in the Text), 551
NEAL, R. A. Enzymic proteolysis by *Entamoeba histolytica*: biochemical characteristics and relationship with invasiveness. (With 10 Figures in the Text), 531
NEAL, R. A. *See* VINCENT, PATRICIA

OSBORNE, P. Observations on the natural enemies of *Meligethes aeneus* (F.) and *M. viridescens* (F.) [Coleoptera: Nitidulidae] (With 16 Figures in the Text), 91
OWEN, I. L. *See* LLEWELLYN, J.

PANDE, B. P., AHLUWALIA, S. S. and SRIVASTAVA, J. S. Note on host-parasite relationships observed in fluke infections of wild aquatic birds. (With 5 Figures in the Text), 323
PANTELOURIS, E. M. and GRESSON, R. A. R. Autoradiographic studies on *Fasciola hepatica* L. (With 1 Plate), 165
PRINGLE, G. Invasion of the egg masses of the mollusc *Bulinus* (*Physopsis*) *globosus*, by larval chironomids, 497

RAWSON, DILYS and RIGBY, JOYCE E. The functional anatomy of the cysticercoid of *Choanotaenia crassiscolex* (Linstow, 1890) (Dilepididae) from the digestive gland of *Oxychilus cellarius* (Müll) (Stylommatophora), with some observations on developmental stages. (With 1 Plate and 5 Figures in the Text), 453
REES, B. *Albertia vermicularis* (Rotifera) parasitic in the earthworm *Allolobophora caliginosa*. (With 1 Plate and 4 Figures in the Text), 61

RIGBY, JOYCE E. *See* RAWSON, DILYS
ROGERS, W. P. and SOMMERVILLE, R. I. The physiology of the second ecdysis of parasitic nematodes. (With 1 Plate and 11 Figures in the Text), 329

SEAMER, J. Studies with *Eperythrozoon parvum* Splitter, 1950. (With 1 Figure in the Text), 67
SOLTYS, M. A. *See* INGRAM, D. G.
SOMMERVILLE, R. I. The growth of *Cooperia curticei* (Giles, 1892), a nematode parasite of sheep. (With 2 Figures in the Text), 261
SOMMERVILLE, R. I. *See* ROGERS, W. P.
SPRENT, J. F. A. and MINES, J. J. A new species of *Amplicaecum* (Nematoda) from the carpet snake (*Morelia argus variegatus*): with a re-definition and a key for the genus. (With 20 Figures in the Text), 183
SRIVASTAVA, J. S. *See* PANDE, B. P.
STEVENS, A. J. *See* THOMAS, R. J.

TATCHELL, R. J. A comparative account of the tracheal system of larvae of the horse bot-fly, *Gasterophilus intestinalis* (De Geer), and of some other dipterous larvae. (With 16 Figures in the Text), 481
TATE, P. Notes on *Limnophora exsurda* Pand. (Diptera, Anthomyidae). (With 7 Figures in the Text), 527
THEODOR, O. and COSTA, M. New species and new records of Argasidae from Israel. Observations on the rudimentary scutum and the respiratory system of the larvae of the Argasidae. (With 28 Figures in the Text), 365
THOMAS, R. J. and STEVENS, A. J. Ecological studies on the development of the pasture stages of *Nematodirus battus* and *N. filicollis*, nematode parasites of sheep. (With 5 Figures in the Text), 31

VICKERMAN, K. *Herpetomonas ludwigi* (Kramář, 1950) n.comb., the trypanosomatid parasite of cranefly larvae (Diptera, Tipulidae). (With 5 Figures in the Text), 351
VINCENT, PATRICIA and NEAL, R. A. Duration of invasiveness of *Entamoeba histolytica* maintained *in vitro*. (With 1 Figure in the Text), 449

WALTON, G. A. The reaction of some variants of *Ornithodoros moubata* Murray (Argasidae, Ixodoidea) to desiccation. (With 2 Figures in the Text), 81
WILLIAMS, H. HARFORD. Some observations on *Parabothrium gadi-pollachii* (Rudolphi, 1810) and *Abothrium gadi* van Beneden 1870 (Cestoda: Pseudophyllidea) including an account of their mode of attachment and of variation in the two species. (With 40 Figures in the Text), 303
WILLIAMS, H. HARFORD. *See* EUZET, L.

Index of Subjects

Abothrium gadi, 303
Albertia vermicularis, parasitic in the intestine of *Allolobophora caliginosa* in Wales, 61
Allolobophora caliginosa, host of *Albertia vermicularis* in Wales, 61
Amplicaecum, key to the genus, 183
Amplicaecum robertsi, parasitic in the carpet snake, re-description of, 183
Anisus natalensis, development of the trematode *Carmyerius exoporus* in, 469
Antibiotics, effects of, on symbionts of *Rhodnius prolixus*, 279
Antibodies, cytoplasm-modifying, nature of, in mice infected with *Toxoplasma*, 419
Apatemon gracilis minor, development of, in molluscs and leeches, 401
Apodemus speciosus peninsulae, host of the mite *Shunsennia gracilis* in Korea, 577
Aquatic birds, fluke infections of, in India, 323
Argas, morphology and systematics of Israelian species of, 365
Argasidae, new species and records of, in Israel, 365
Artyfechinostomum, key to the species of, 1
Artyfechinostomum mehrai, morphology of, 7
Autoradiographic studies on the intestinal epithelium of *Fasciola hepatica*, 165

Birds, Indian aquatic, fluke infections of, 323
Blatta orientalis, host of *Hammerschmidtiella diesingi*, 241
Bulinus (*Physopsis*) *globosus*, egg masses of, infected by chironomid larvae, in Tanganyika, 497

Caleocotyle palombi, description of, 21
Caleocotyle stossichii, re-description of, 21
Carmyerius exoporus, development of, in snails in Kenya, 469
Carpet snake, host of the nematode *Amplicaecum*, 183
Cathaemasia spp. in Indian aquatic birds, 323
Cercaria brevis, in *Lymnaea stagnalis*, identical with *Cercaria helvetica*, 551
Cercaria spp. in snails from Edgbaston Pool, Birmingham, 537
Cestodes, secretions of Mehlis's gland of, 349
Chironomid larvae, invading egg masses of *Bulinus* (*Physopsis*) *globosus* in Tanganyika, 497
Chironomus plumosus, larvae of, as hosts of the ciliate *Tetrahymena chironomi*, 111
Choanotaenia crassiscolex, anatomy and development of the cysticercoid in *Oxychilus cellarius*, 453
Cooperia curticei, growth of, in sheep, 261
Cotylurus brevis, life history of, 551
Crane fly larvae, hosts of *Herpetomonas ludwigi*, 351
Cytoplasm-modifying antibodies, nature of, in mice infected with *Toxoplasma*, 419

Dipterous larvae, tracheal system of, 481
Discocotyle sagittata, mode of attachment to the gills of *Salmo trutta* in Britain, 351
Dactylosoma, species of, in freshwater fish in Africa, 515

Ecdysis, second, of parasitic nematodes, physiology of, 329
Echinochasmus, species of, in Indian aquatic birds, 323
Eimeria stiedae, lipids in, 501
Entamoeba histolytica, duration of invasiveness of *in vitro*, 449
  enzyme proteolysis by and invasiveness of, 531
Eperythrozoon parvum, structure, epidemiology, effects on pigs and susceptibility to drugs, 67

Fasciola hepatica, autoradiographic studies on the intestinal epithelium of, 165
Fat, distribution of, in *Thelastoma bulhõesi*, 247
  in *Hammerschmidtiella diesingi*, 247
Fluke infestations of Indian aquatic birds, 323

Freshwater, fish in East Africa, hosts of *Trypanosoma mukasai* and *Dactylosoma mariae*, 515
Furcocercariae, species of, in freshwater molluscs in Nyasaland, 401

Gasterophilus intestinalis, tracheal system of, 481
Geckobia japonicum, identical with *Pterygosoma japonicum*, 89
Glycogen, distribution of, in *Thelastoma bulhõesi*, 247

Hammerschmidtiella diesingi, distribution of fat in, 247
  osmotic pressures in, in relation to its survival in the hind gut of *Blatta orientalis*, 241
Hepatocystis kochi, lipids in, 501
  polysaccharides in, 509
Hepatozoon spp., lipids in, 501
  polysaccharides in, 509
Herpetomonas ludwigi, in crane fly larvae, 351

Indian rhynchobdellid leeches, 287
Immuno-conglutinin in rabbits and mice infected with *Trypanosoma brucei*, 231
Intestinal epithelium, metabolism of, in *Fasciola hepatica*, 165
Invasiveness of *Entamoeba histolytica*, 449, 531
Ixodes auritulus group of Ixodidae, taxonomy of, 199
Ixodes auritulus-percavatus, taxonomy of, 199
Ixodes cornuae, description of, 199
Ixodes kerguelenensis, re-description of, 227
Ixodes percavatus group of Ixodidae, taxonomy of, 199
Ixodes pterodromae, description of, 199

Lallum magniparuterina (Cestoda) in the intestine of *Nettion crecca*, description of, 269
Lankesterella, polysaccharides in, 509
Larval trematodes of freshwater molluscs in Nyasaland, 401
Leeches, Indian rhynchobdellid, 287
  freshwater, metacercariae in, 401
  hosts of metacercariae, 401
Limnophora exsurda, eggs, larvae and nomenclature of, 527
Lipids, in species of *Plasmodium*, *Hepatocystis*, *Hepatozoon* and *Eimeria*, 501.

Mehlis's gland of trematodes and cestodes, secretions of, 349
  of *Penetrocephalus ganapatii*, 155
Meligethes aeneus, protozoan, hymenopterous, ichneumonid and other parasites of, 91
Meligethes viridescens, parasites and predators of, 91
Metacercariae, in freshwater leeches, 401
Morelia argus variegatus, host of the nematode *Amplicaecum*, 183
Mouse, Australian pouched, host of Trichostrongylidae, 425

Nematodes, parasitic, second ecdysis, physiology of, 329
Nematodirus battus, ecology of the larvae of, on pastures, 31
Nematodirus filicollis, ecology of the larvae of, on pastures, 31
Neocardia rhodnii, symbiotic with *Rhodnius prolixus*, sensitivity of, to antibiotics, 279
Nephridiophaga xeonoboli, microsporidian in the Indian millipede *Xenobolus carnifex*, 581
Nettion crecca, host of *Lallum magniparuterina*, 269
Nicollina phascogale, in the Australian pouched mouse, *Phascogale flavipes*, 425
Nutrition experiments on sheep, importance of spring rise of faecal worm egg counts in, 387

Opalina, encystation of, correlated with the reproductive cycle of *Rana temporaria*, 171
Ornithodoros, morphology and systematics of Israelean species of, 365
Ornithodoros lahorensis, species and larval respiratory system of, 365
Ornithodoros moubata, reaction of African variants to desiccation, 81
Osmotic pressure, effect of, on the survival of *Hammerschmidtiella diesingi*, 241
Oxychilus cellarius, intermediate host of *Choanotaenia crassiscolex*, 453

Parabothrium gadi-pollachii, description of and differentiation from *A. gadi*, 303
Parasites and predators of *Meligethes aeneus*, 91
Parasitic nematodes, second ecdysis, physiology of, 329
Penetrocephalus ganapatii (Cestoda), in the teleostean Indian fish, *Saurida tumbil*, 155
secretions of Mehlis's gland of, 349
Phascogale flavipes, host of Trichostrongylidae in Australia, 425
Physopsis (*Bulinus*) *globosus*, egg masses of, invaded by chironomid larvae in Tanganyika, 497
Pigs, effects of *Eperythrozoon parvum* on, 67
Pirhaemocyton sp., polysaccharides in, 509
Plasmodium cynomolgi, lipids in, 501
polysaccharides in, 509
Plasmodium gallinaceum, development of the gametocytes *in vitro*, 431
lipids in, 501
polysaccharides in, 509
Polypedilum (*Polypedilum*) *kibatiense* (Chironomidae), larvae of, invading the egg masses of *Bulinus* (*Physopsis*) *globosus*, 497
Polysaccharides, in Sporozoa, 509
Proteolysis of *Entamoeba histolytica*, 531
Pterygosoma japonicum, identity of with *Geckobia japonicum*, 89

Rana temporaria, reproductive cycle correlated with encystment of *Opalina*, 171
Rhodnius prolixus, apparatus for the artificial feeding of, 273
growth and development of, with and without symbionts, 279
Rhynchobdellid leeches, Indian, 287

Sabin-Feldman phenomenon in *Toxoplasma*, indicator system of, 13
Salmo trutta, host of *Discocotyle sagittata* (Monocotylea) in Britain, 51
Saurida tumbil, host of *Penetrocephalus ganapatii* (Cestoda), 155
Sea birds, species of *Ixodes* on, 199, 227
Sheep, infection of in the spring, but not in the summer, by *Nematodirus battus* and *N. filicollis*, 31
Shrew, host of *Choanotaenia crassiscolex*, in Britain, 453
Shunsennia gracilis, trombiculid mite from the woodmouse in Korea, 577
Snails, from Edgbaston Pool, Birmingham, cercariae in, 551
Sorex araneus, host of *Choanotaenia crassiscolex* in Britain, 453
Sporozoa, lipids in, 501
polysaccharides in, 509
Spring rise of worm eggs counts of sheep, importance of, in nutritional experiments, 387

Tetrabothriostrongylus mackerrasae, in the Australian pouched mouse, *Phascogale flavipes*, 425
Tetracotyle, species of, in freshwater leeches, 401
Tetrahymena, facultative parasitism of the genus, 111
Tetrahymena chironomi, systematics, structure and parasitism in larvae of *Chironomus plumosus*, 111
Theileria parva, polysaccharides in, 509
Thelastoma bulhõesi, distribution of fat and glycogen in, 247
parasitic in *Periplaneta orientalis*, 247
Toxoplasma, indicator system of Sabin-Feldman phenomenon in, 13
Tracheal system of *Gasterophilus intestinalis* and other dipterous larvae, 481
Trematode larvae of freshwater molluscs in Nyasaland, 401
Trematodes, secretion of Mehlis's gland of, 349
Triatoma infestans, symbionts of, 279
Trichostrongylidae, two species of in the Australian pouched mouse, *Phascogale flavipes*, 425
Trypanosoma brucei, immuno-conglutinin in rabbits and mice infected with, 231
Trypanosoma mukasai, in freshwater fish of East Africa, 515

Water birds seen at Edgbaston Pool, Birmingham, 551
Worm egg counts of sheep, importance of spring rise of, in nutritional experiments, 387

Xenobolus carnifex, Indian millipede host of *Nephridiophaga xenoboli*, 581

Vol. 50, Nos. 3, 4 November 1960

JUL 7 1964
MEDICAL LIBRARY

PARASITOLOGY

(Founded by G. H. F. Nuttall)

Medical Library
WA
P22
V.50

EDITED BY

D. KEILIN and P. TATE

IN CONJUNCTION WITH

A. BISHOP
F. J. BROWN
B. DAWES
G. O. EVANS
E. HINDLE
C. A. HOARE
G. LAPAGE
E. W. McCONNACHIE
B. G. PETERS
G. REES
J. SMART
K. M. SMITH

CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W. 1
AMERICAN BRANCH: 32 EAST 57TH STREET, NEW YORK 22, N.Y

Fifty-five Shillings net
(U.S.A. $10.50)

A list of the

JOURNALS

Published by the Cambridge University Press, may be had from

THE MANAGER
CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE
200 EUSTON ROAD
LONDON, N.W. 1

MICHROME STAINS

and Reagents for Microscopy

Adenosine	Gallocyanin	Methyl Green
Adenylic acids	Giemsa Stain	Ribonuclease
Celloidin	Janus Green, B	Sudan Black
Fluormount	Leishman Stain	TPN
Fluoroil	Luxol Fast Blue	Urease

OPTOIL: synthetic, non-sticky immersion oil.

TRIFALGIC ACID, for cytology, electrophoresis, etc. (Ref. Irish J.Med.Sc., April 1960, pages 182/6)

62-page catalogue available on request

Service, uniform high quality, reliability, and immediate delivery

NOW READY: *Books by Edward Gurr:*

ENCYCLOPAEDIA OF MICROSCOPIC STAINS, 500 pages Royal 8vo, price 95/-.

"Methods of Analytical Histology & Histochemistry" 342 pages, Royal 8vo, price 70/-.

"Microscopic Staining Techniques", No. 4 (1st edit.), 66 pages, price 6/- ($1.00 in U.S.A.), No. 3 (2nd edit.), 66 pages, 6/- ($1.00 in U.S.A.).

"A Practical Manual of Medical & Biological Staining Techniques", 460 pages, 42/-, Postage 2/6.

EDWARD GURR, LTD.
42 Upper Richmond Road West
LONDON, S.W.14

Publications issued by

COMMONWEALTH INSTITUTE OF ENTOMOLOGY
56 Queen's Gate, London, S.W.7

BULLETIN OF ENTOMOLOGICAL RESEARCH

Published quarterly and containing original articles on Economic Entomology. *Issued Post Free.*
Annual Subscription (payable in advance) 100s. 0d.
Prices of back parts and volumes on application.

REVIEW OF APPLIED ENTOMOLOGY

Consisting of abstracts or reviews of current literature on Economic Entomology throughout the world. Published monthly in two series:

Series "A" dealing with insect and other Arthropod pests of cultivated plants, forest trees and stored products of animal and vegetable origin.

Series "B" dealing with insects, ticks, etc. conveying disease or otherwise injurious to man and animals. *Issued Post Free.*

	Series "A"	*Series "B"*
Annual Subscription (payable in advance)	60s. 0d.	30s. 0d.

Prices of back parts and volumes on application.

ZOOLOGICAL RECORD, part INSECTA

Published annually about October each year and containing as complete a record as possible of the literature of the previous year, chiefly from the systematic standpoint.
Annual Subscription (including postage) 61s. 6d.
Prices of back volumes on application.

The Journal of General Microbiology

Editors: B. C. J. G. KNIGHT and A. F. B. STANDFAST

Contents Volume 23, No. 3, December 1960

PINE, L., HOWELL, A. Jnr. and WATSON, S. J. Studies on the morphological, physiological and biochemical character of *Actinomyces bovis*.

TRISTRAM, H. The adaptive degradation of L-histidine by *Paracolobactrum aerogenoides*.

KRISS, A. E. and MITSKEVICH, I. N. The distribution of filamentous-cluster organisms (Krassilnikoviae) in sea and ocean depths.

COETZEE, J. N. and SACKS, T. G. Transduction of streptomycin resistance in *Proteus mirabilis*.

BAUCHOP, T. and ELSDEN, S. R. The growth of micro-organisms in relation to their energy supply.

BROWN, A. D. Some properties and a proposed classification of a Gram-negative heterotrophic marine bacterium.

LASCELLES, J. The synthesis of enzymes concerned in bacteriochlorophyll formation in growing cultures of *Rhodopseudomonas spheroides*.

LASCELLES, J. The formation of ribulose 1:5-diphosphate carboxylase by growing cultures of Athiorhodaceae.

HILL, R. F. and SIMSON, E. A study of radio-sensitive and radio-resistant mutants of *Escherichia coli* strain B.

KERRIDGE, D. The effect of inhibitors on the formation of flagella by *Salmonella typhimurium*.

POSTGATE, J., CRUMPTON, J. and HUNTER, J. R. Measurement of bacterial viabilities by slide culture.

KORNBERG, H. L. and LASCELLES, J. The formation of isocitratase by the Athiorhodaceae.

MUKAI, F. H. Inter-relationship between colicin sensitivity and phage resistance.

BRADLEY, D. E. and KAY, D. The fine structure of bacteriophages.

KISTNER, A. An improved method for viable counts of bacteria fermenting various carbohydrates in the ovine rumen.

KARSKA, B., KOZAK, W., NARBUTOWICZ, B. and STOLARSKA, A. Study on the lysogenicity of staphylococci isolated in Poland.

MARINI, F., ARNOW, P. and LAMPEN, J. O. Effect of monovalent cations on the inhibition of yeast metabolism by nystatin.

TAYLOR, J., LEE, M. M., EDWARDS, P. R. and RAMSEY, C. H. A new type of flagellar variation associated with new antigens in the salmonella group.

WEITZ, B. The properties of some antigens of *Trypanosoma brucei*.

COWAN, S. T., STEEL, K. J., SHAW, C. and DUGUID, J. P. A classification of the Klebsiella group.

FURNESS, G., GRAHAM, D. M. and REEVE, P. The Titration of Trachoma and Inclusion Blennorrhoea Viruses in Cell Cultures.

The subscription rate is 90s. net per volume of 3 parts. Single parts 35s. each, plus postage. Two volumes will be issued each year. Orders should be sent to

CAMBRIDGE UNIVERSITY PRESS

BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W.1

PARASITOLOGY is published in double issues twice a year. The numbers afterwards are issued in volumes each containing four numbers.

Papers for publication should be sent to Professor D. KEILIN, Molteno Institute, Downing Street, Cambridge. Other communications should be addressed to the University Press, Cambridge.

Papers forwarded to the Editors for publication are understood to be offered to *PARASITOLOGY* alone, unless the contrary is stated.

Contributors receive fifty copies of their papers free. Additional copies, not exceeding one hundred (except in special cases), may be had at cost price: these should be ordered when the final proof is returned.

The subscription price, payable in advance, is £5 net per volume, post-free (U.S.A. $19.50), double numbers 55*s*. net, postage extra (U.S.A. $10.50). Subscriptions may be sent to any Bookseller, or to The Cambridge University Press, Bentley House, 200 Euston Road, N.W. 1.

Enquiries from the U.S.A. should be addressed to the American Branch of the Press at 32 East 57th Street, New York, 22.

CONTENTS

(*All rights reserved*)

PAGE

BAUGH, SANJIB CHANDRA. Studies on Indian Rhynchobdellid leeches. I. (With 6 Figures in the Text) 287

WILLIAMS, H. HARFORD. Some observations on *Parabothrium gadi-pollachii* (Rudolphi, 1810) and *Abothrium gadi* van Beneden 1870 (Cestoda: Pseudophyllidea) including an account of their mode of attachment and of variation in the two species. (With 40 Figures in the Text) 303

PANDE, B. P., AHLUWALIA, S. S. and SRIVASTAVA, J. S. Note on host-parasite relationships observed in fluke infections of wild aquatic birds. (With 5 Figures in the Text) 323

ROGERS, W. P. and SOMMERVILLE, R. I. The physiology of the second ecdysis of parasitic nematodes. (With 1 Plate and 11 Figures in the Text) 329

HANUMANTHA-RAO, K. The problem of Mehlis's gland in helminths with special reference to *Penetrocephalus ganapatii* (Cestoda: Pseudophyllidea) 349

VICKERMAN, K. *Herpetomonas ludwigi* (Kramář, 1950) n.comb., the trypanosomatid parasite of cranefly larvae (Diptera, Tipulidae). (With 5 Figures in the Text) . 351

THEODOR, O. and COSTA, M. New species and new records of Argasidae from Israel. Observations on the rudimentary scutum and the respiratory system of the larvae of the Argasidae. (With 28 Figures in the Text) 365

FIELD, A. C., BRAMBELL, M. R. and CAMPBELL, J. ALLAN. Spring rise in faecal worm-egg counts of housed sheep, and its importance in nutritional experiments. (With 3 Figures in the Text) 387

ILES, CHRISTINE. The larval trematodes of certain fresh-water molluscs. (With 1 Plate) 401

KULASIRI, C. The nature of the cytoplasm-modifying antibodies. (With 2 Figures in the Text) 419

MAWSON, PATRICIA M. Two new species of trichostrongyle nematodes from the Australian pouched mouse, *Phascogale flavipes* (Waterhouse). (With 11 Figures in the Text) 425

BISHOP, ANN and MCCONNACHIE, ELSPETH W. Further observations on the *in vitro* development of the gametocytes of *Plasmodium gallinaceum* 431

VINCENT, PATRICIA and NEAL, R. A. Duration of invasiveness of *Entamoeba histolytica* maintained *in vitro*. (With 1 Figure in the Text) 449

RAWSON, DILYS and RIGBY, JOYCE E. The functional anatomy of the cysticercoid of *Choanotaenia crassiscolex* (Linstow 1890) (Dilepididae) from the digestive gland of *Oxychilus cellarius* (Müll) (Stylommatophora), with some observations on developmental stages. (With 1 Plate and 5 Figures in the Text) . . . 453

DINNIK, J. A. and DINNIK, N. N. Development of *Carmyerius exoporus* Maplestone (Trematoda: Gastrothylacidae) in a snail host. (With 8 Figures in the Text) . 469

TATCHELL, R. J. A comparative account of the tracheal system of larvae of the horse bot-fly, *Gasterophilus intestinalis* (De Geer), and of some other dipterous larvae. (With 16 Figures in the Text) 481

PRINGLE, G. Invasion of the egg masses of the mollusc *Bulinus* (*Physopsis*) *globosus*, by larval chironomids 497

DASGUPTA, B. Lipids in different stages of the life cycles of malaria parasites and some other sporozoa. (With 2 Figures in the Text) 501

DASGUPTA, B. Polysaccharides in the different stages of the life-cycles of certain sporozoa. (With 3 Figures in the Text) 509

BAKER, J. R. Trypanosomes and dactylosomes from the blood of fresh-water fish in East Africa. (With 1 Plate and 2 Figures in the Text) 515

TATE, P. Notes on *Limnophora exsurda* Pand. (Diptera, Anthomyidae). (With 7 Figures in the Text) 527

NEAL, R. A. Enzymic proteolysis by *Entamoeba histolytica*: biochemical characteristics and relationship with invasiveness. (With 10 Figures in the Text) . 531

NASIR, P. Trematode parasites of snails from Edgbaston Pool: the life history of the strigeid *Cotylurus brevis* Dubois & Rausch, 1950. (With 8 Figures in the Text) 551

AH, HYONG-SUN. *Shunsennia gracilis*, a new species of Trombiculid Mite from Korea (Acarina, Trombiculidae). (With 1 Figure in the Text) 577

GANAPATI, P. N. and NARASIMHAMURTI, C. C. On a new haplosporidian (protozoa) *Nephridiophaga xenoboli* in the gut of a millipede *Xenobolus carnifex*. (With 9 Figures in the Text) 581

Index of Authors 586

Index of Subjects 589

Printed in Great Britain at the University Press, Cambridge
(*Brooke Crutchley, University Printer*)

4538